Atty. Dkt. No. 017227/0155

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: John C. COX et al

Title: IMMUNOGENIC COMPLEXES AND METHODS RELATING THERETO

Appl. No.: UNASSIGNED

Filing Date: February 17, 2000

Examiner: Not yet assigned

Art Unit: Not yet assigned

UTILITY PATENT APPLICATION TRANSMITTAL

Assistant Commissioner for Patents
Box PATENT APPLICATION
Washington, D.C. 20231

Sir:

Transmitted herewith for filing under 37 C.F.R. § 1.53(b) is the nonprovisional utility patent application of:

John Cooper COX
Debbie Pauline DRANE

Enclosed are:

[X] Specification, Claim(s), and Abstract (53 pages).

[X] Informal drawings (20 sheets, Figures 1-15).

[X] Unexecuted Declaration and Power of Attorney (3 pages).

[X] Preliminary Amendment (4 pages).

The filing fee is calculated below:

	Claims as Filed		Included in Basic Fee		Extra Claims		Rate		Fee Totals
Basic Fee							$690.00		$690.00
Total Claims:	44	-	20	=	24	x	$18.00	=	$432.00
Independents:	2	-	3	=	0	x	$78.00	=	$0.00
If any Multiple Dependent Claim(s) present:						+	$260.00	=	$0.00
Surcharge Fee under 37 C.F.R. 1.16(e)						+	$130.00	=	$130.00
							SUBTOTAL:	=	$1,252.00
[ ] Small Entity Fees Apply (subtract ½ of above):								=	$0.00
							TOTAL FILING FEE:	=	$1,252.00

[ ] A check in the amount of $-0- to cover the filing fee is enclosed.

[**X**] The required filing fees are not enclosed but will be submitted in response to the Notice to File Missing Parts of Application.

[ ] The Assistant Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Assistant Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741.

Please direct all correspondence to the undersigned attorney or agent at the address indicated below.

Respectfully submitted,

Date February 17, 2000

By _____

Stephen A. Bent
Attorney for Applicant
Registration No. 29,768

FOLEY & LARDNER
Washington Harbour
3000 K Street, N.W., Suite 500
Washington, D.C. 20007-5109
Telephone: (202) 672-5404
Facsimile: (202) 672-5399

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re patent application of John Cooper Cox et al.

Serial No. UNASSIGNED

Filed: February 17, 2000

For: **IMMUNOGENIC COMPLEXES AND METHODS RELATING THERETO**

PRELIMINARY AMENDMENT

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Prior to examination of the above-identified application, Applicant respectfully requests that the following amendments be entered into the application:

IN THE CLAIMS:

Please cancel claims 44 through 51 without prejudice or disclaimer.

Claim 9, line 1, delete "any one of claims 5-8" and insert --claim 5--;

Claim 8, (second occurrence), line 1, delete "8." and insert --10.--; and delete "any one of claims 5-8" and insert --claim 5--;

Claim 12, line 1, delete "any one of claims 5-8" and insert --claim 5--;

Claim 17, line 1, delete "any one of claims 1-16" and insert --claim 1--;

Claim 26, line 1, delete "any one of claims 22-25" and insert --claim 22--;

Claim 27, line 1, delete "any one of claims 22-25" and insert --claim 22--;

Claim 29, line 1, delete "any one of claims 22-25" and insert --claim 22--;

Claim 34, line 1, delete "any one of claims 18-33" and insert --claim 18--;

Claim 35, line 3, delete "any one of claims 1-17" and insert --claim 1--;

Claim 36, line 3, delete "any one of claims 18-34" and insert --claim 18--;

Claim 37, line 1, delete "17 or";

Claim 38, line 3, delete "any one of claims 1-17" and insert --claim 1--;

Claim 39, line 3, delete "any one of claims 18-34" and insert --claim 18--;

Claim 40, line 1, delete "or 39";

Claim 41, line 1, delete "any one of claims 38-41" and insert --claim 38--;

Claim 42, line 1, delete "any one of claims 38-41" and insert --claim 38--;

Please add the following new claim:

--52. The method according to claim 39 wherein said treatment is therapeutic or prophylactic.--

REMARKS

Applicants respectfully request that the foregoing amendments to Claims 9, 10, 12, 17, 26, 27, 29, 34-42, 44-51 and therefore adding new Claim 52 be entered. These amendments are being made in order to avoid this application incurring a surcharge for the presence of one or more multiple dependent claims. No new matter has been added.

Respectfully submitted,

February 17, 2000
Date

Stephen A. Bent
Reg. No. 29,768

FOLEY & LARDNER
Washington Harbour
3000 K Street, N.W., Suite 500
Washington, DC 20007-5109

IMMUNOGENIC COMPLEXES AND METHODS RELATING THERETO

FIELD OF THE INVENTION

The present invention relates generally to an immunogenic complex comprising a charged organic carrier and a charged antigen and, more particularly, a negatively charged organic carrier and a positively charged antigen. The complexes of the present invention are useful, *inter alia*, as therapeutic and/or prophylactic agents for facilitating the induction of a cytotoxic T-lymphocyte response to an antigen.

BACKGROUND OF THE INVENTION

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

There is an increasing belief that co-delivery of antigen and adjuvant to the same antigen-presenting-cell (APC) is preferable and sometimes essential for induction of appropriate immune responses. For example, the ability of saponin-based adjuvants to induce CD_8^+ CTL responses is attributed to their ability to cause endosomal escape of antigen, a mechanism which requires co-delivery. Particle formation which comprises a stable complex of adjuvant and antigen is the simplest way to achieve co-delivery. The usefulness of ISCOM™ technology derives partly from the immunomodulatory activity of saponins and partly from their ability to form complexes with hydrophobic or amphipathic immunogens. However, many molecules lack hydrophobic regions and in fact such molecules are preferred as recombinant proteins because of their easier expression and purification.

Accordingly, there is a need to develop immunogenic complexes which facilitate the co-delivery of antigens and carriers which otherwise do not usually form sufficiently stable complexes. For example, complexes comprising antigens which lack hydrophobic regions together with adjuvant.

In work leading up to the present invention, the inventors have developed an immunogenic complex based on the electrostatic association of an antigen and an organic carrier, such as an adjuvant. This electrostatic association permits co-delivery of the antigen and the organic carrier to the immune system. Accordingly, by establishing an electrostatic association, antigens of interest (irrespective of their hydrophobicity) can be co-delivered with an organic carrier , for the purpose, for example, of inducing a cytotoxic T-lymphocyte response to the antigen.

SUMMARY OF THE INVENTION

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The subject specification contains amino acid sequence information prepared using the programme PatentIn Version 2.0, presented herein after the bibliography. Each amino acid sequence is identified in the sequence listing by the numeric indicator <210> followed by the sequence identifier (e.g. <210>1, <210>2, etc.). The length, type of sequence (protein (PRT), etc) and source organism for each amino acid sequence are indicated by information provided in the numeric indicator fields <211>, <212> and <213>, respectively. Amino acid sequences referred to in the specification are defined by the information provided in numeric indicator field <400> followed by the sequence identifier (e.g. <400>1, <400>2, etc).

One aspect of the present invention relates to an immunogenic complex comprising a charged organic carrier and a charged antigen which organic carrier and antigen are electrostatically associated.

Another aspect of the present invention more particularly provides an immunogenic complex comprising a negatively charged organic carrier and a positively charged antigen which organic carrier and antigen are electrostatically associated.

Still another aspect of the present invention provides an immunogenic complex comprising a negatively charged organic carrier and a positively charged protein which organic carrier and protein are electrostatically associated.

Yet another aspect of the present invention provides an immunogenic complex comprising a negatively charged adjuvant and a positively charged protein which adjuvant and protein are electrostatically associated.

Yet still another aspect of the present invention provides an immunogenic complex comprising a negatively charged adjuvant and a positively charged protein, wherein said negatively charged adjuvant is a naturally negatively charged adjuvant which has been modified to increase the degree of its negative charge, which adjuvant and protein are electrostatically associated.

Still another aspect of the present invention provides an immunogenic complex comprising a negatively charged adjuvant and a positively charged protein, wherein said positively charged protein is a naturally positively charged protein which has been modified to increase the degree of its positive charge, which adjuvant and protein are electrostatically associated.

Still yet another aspect of the present invention provides an immunogenic complex comprising a negatively charged adjuvant and a positively charged protein, wherein said negatively charged adjuvant is a naturally negatively charged adjuvant which has been modified to increase the degree of its negative charge and said positively charged protein is a naturally positively charged protein which has been modified to increase the degree of its positive charge, which adjuvant and protein are electrostatically associated.

A further aspect of the present invention relates to a vaccine composition comprising as the active component an immunogenic complex comprising a charged organic carrier and a charged antigen which organic carrier and antigen are electrostatically associated together with one or more pharmaceutically acceptable carriers and/or diluent.

Another further aspect of the present invention relates to a method of eliciting, inducing or otherwise facilitating, in a mammal, an immune response to an antigen said method comprising administering to said mammal an effective amount of an immunogenic complex or a vaccine composition as hereinbefore described.

Yet another further aspect of the present invention relates to a method of treating a disease condition in a mammal said method comprising administering to said mammal an effective amount of an immunogenic complex or a vaccine composition as hereinbefore described wherein administering said composition elicits, induces or otherwise facilitates an immune response which inhibits, halts, delays or prevents the onset or progression of the disease condition.

Still another further aspect the present invention relates to the use an immunogenic complex or vaccine composition as hereinbefore defined in the manufacture of a medicament for inhibiting, halting, delaying or preventing the onset or progression of a disease condition.

Still yet another further aspect of the present invention relates to an agent for use in inhibiting, halting, delaying or preventing the onset or progression of a disease condition. Said agent comprising an immunogenic complex or vaccine composition as hereinbefore defined.

Single and three letter abbreviations used throughout the specification are defined in Table 1.

TABLE 1

Single and three letter amino acid abbreviations

Amino Acid	Three-letter Abbreviation	One-letter Symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Any residue	Xaa	X

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graphical representation of the sucrose gradient analysis of ISCOMATRIX™ formulated with DPPC (Figure 1A), CDL (Figure 1B), DPL (Figure 1C), MPL (Figure 1D), DPA (Figure 1E) and DPPG (Figure 1F). In each case it can be seen that lipid and ^{3}H overlap indicating incorporation of each lipid into the ISCOMATRIX™ structure.

Figure 2 is a graphical representation of the sucrose gradient analysis of four of the ISCOMATRIX™ formulations of Example 1 after mixing with HpE. It can be seen that most of the HpE is with CDL and DPL ISCOMATRIX™ but only part is associated with DPPC and DPPG ISCOMATRIX™.

Figure 3 is a graphical representation of the sucrose gradient analysis of two of the ISCOMATRIX™ formulations of Example 1 after mixing with ESO. It can be seen that most of the ESO is associated with DPL ISCOMATRIX™ but only part is associated with DPPC ISCOMATRIX™.

Figure 4 is a graphical representation of antibody responses to ESO formulations. It can be seen that ESO associated ISCOMATRIX™ incudes higher antibody responses than ESO alone especially in the Th1 subtype IgG2a.

Figure 5 is a graphical representation of CTL analysis of mice immunised with ESO (Figures 5A, 5C) and ESO associated ISCOMATRIX™ (Figures 5B, 5D) using SLLMWITQCFL (<400>1)(Figure 5A, 5B) and SLLMWITQC (<400>2) (Figure 5C, 5D) peptides for stimulation and targets. It can be seen that ESO associated ISCOMATRIX™ induces a CTL response but ESO alone does not.

Figure 6 is a graphical representation of the sucrose gradient analysis of six of the ISCOMATRIX™ formulations of Example 1 after mixing with E6E7. It can be seen that most of the E6E7 is associated with CDL, DPL and DPA ISCOMATRIX™, less associated

with the MPL and DPPG ISCOMATRIX™ and even less again with the DPPC ISCOMATRIX™.

Figure 7 is a graphical representation of CTL analysis in mice immunised with E6E7 DPL ISCOMATRIX™ (Figure 7A) and E6E7 DPPC ISCOMATRIX™ (Figure 7B). It can be seen that E6E7 DPL ISCOMATRIX™ induces CTL responses but E6E7 DPPC ISCOMATRIX™ does not.

Figure 8 is a graphical representation of the sucrose gradient analysis of two of the ISCOMATRIX™ formulations from Example 1 after mixing with HpC. It can be seen that more HpC is associated with DPL ISCOMATRIX™ than with DPPC ISCOMATRIX™ where there is very little association.

Figure 9 is a graphical representation of the sucrose gradient analysis of two DPPC ISCOMATRIX™ formulations after mixing with E6E7 at pH6 (Figure 6A) and pH7.2 (Figure 6B). It can be seen that more E6E7 associates with DPPC ISCOMATRIX™ at pH6 than at pH7.2.

Figure 10 is a graphical representation of the sucrose gradient analysis of ISCOMATRIX™ formulations after mixing with modified HpC from Example 11. It can be seen that addition of 6K to HpC increases the association with DPPC ISCOMATRIX™ to a level comparable to that with the 6H and CHL ISCOMATRIX™ formulation.

Figure 11 is a graphical representation of the sucrose gradient analysis of four polytope ISCOM™ and ISCOMATRIX™ formulations from Example 13. It can be seen that there is some association of the 6K polytope with ISCOMATRIX™ but there is no association if the 6K are not present. The 6K polytope association with ISCOMATRIX™ was comparable to hydrophobic incorporation of the PAL polytope into ISCOMs™ but less than association between 6H polytope and CHL ISCOMATRIX™.

Figure 12 is a graphical representation of the CTL analysis of four synthetic polytope ISCOMATRIX™ formulations from Example 13. It can be seen that 6K polytope ISCOMATRIX™ induced CTL responses against all 4 epitopes in the polytope (Figure 12C) but the polytope ISCOMATRIX™ formulation without a tag only induced a low CTL response to one of the epitopes (Figure 12D). The CTL responses for the 6K polytope ISCOMATRIX™ were comparable to those induced with the PAL polytope ISCOM™ (Figure 12A) and the 6H polytope CHL ISCOMATRIX™ (Figure 12B).

Figure 13 is a graphical representation of the sucrose gradient analysis of ten recombinant ISCOMATRIX™ formulations from Example 16. It can be seen that the combination of adding a 6K tag with CDL or DPL ISCOMATRIX™ gives increased association over 6K with DPPC ISCOMATRIX™ and to then combine these with low pH increase the capacity to associate even further. The association achieved with the combination of 6K, CDL ISCOMATRIX™ and low pH gave almost complete association of the polytope with ISCOMATRIX™ and the association was greater than could be achieved with 6H polytope CHL ISCOMATRIX™.

Figure 14 is a graphical representation of the CTL analysis of the 6K polytope CDL ISCOMATRIX™ pH4.3 (Figure 14A) and 6H polytope CHL ISCOMATRIX™ (Figure 14B) formulations. It can be seen that CTL responses were induced to all 4 epitopes in the polytope for both the formulations but the responses were very low to the TYQ epitope.

Figure 15 is a graphical representation of the liposomes mixed with E6E7 from Example 18. It can be seen that most of the E6E7 was associated with the DPL liposomes but very little E6E7 was associated with the DPPC liposomes.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is predicated, in part, on the development of an immunogenic complex formulation which utilises electrostatic interactions to associate an antigen and a carrier thereby facilitating, *inter alia*, the co-delivery of these molecules to the immune system. The immunogenic complexes of the present invention are particularly suitable for use in facilitating the stimulation of cytotoxic T-lymphocyte responses to immunogens which do not comprise hydrophobic regions.

Accordingly, one aspect of the present invention relates to an immunogenic complex comprising a charged organic carrier and a charged antigen which organic carrier and antigen are electrostatically associated.

Reference to a "complex" should be understood as describing an entity of two or more different interacting chemical components.

Reference to a "charged" organic carrier or antigen should be understood as a reference to an organic carrier or antigen which exhibits an overall positive electrical charge or an overall negative electrical charge. By "overall" is meant the summation of the individual positive and negative charges which a given molecule comprises. Where the summation of the individual positive and negative charges results in overall electrical neutrality, the molecule is not regarded as "charged" within the context of the present invention. Preferably, the antigen comprises an overall positive charge and the organic carrier comprises an overall negative charge.

Accordingly, the present invention more particularly provides an immunogenic complex comprising a negatively charged organic carrier and a positively charged antigen which organic carrier and antigen are electrostatically associated.

Reference to "electrostatically associated" is a reference to the organic carrier and the antigen being linked, bound or otherwise associated by means which include electrostatic

interaction. Accordingly, it should be understood that in some instances the electrostatic interaction will be the only attractive force which results in complexing of the antigen and the organic carrier. However, in other instances the formation of the electrostatic interaction may also lead to, or be associated with, the formation of other interactive forces.

Reference to "antigen" should be understood as a reference to any molecule against which it is sought to induce an immune response, and in particular, a cytotoxic T-lymphocyte response. The antigen may be either a proteinaceous or a non-proteinaceous molecule, which molecule may or may not be immunogenic if it were administered in isolation. The antigen of the present invention may be naturally derived or it may be recombinantly or synthetically produced. Following its isolation or synthesis the antigen may require further modification (for example, structural or sequence modification to improve its antigenicity) prior to use in the present invention. Antigens suitable for use in the present invention include, but are not limited to, core proteins or nucleoproteins isolated from viruses, non-core viral proteins such as virus-like particles (VLPs), antigens of malignant and non-malignant cells, bacterial antigens, parasite antigens and synthetic and recombinant polytopes.

Preferably, the antigen is a protein. The term "protein" should be understood to encompass reference to proteins, polypeptides and peptides and derivatives and equivalents thereof. The protein may be glycosylated or unglycosylated, phosphorylated or dephosphorylated to various degrees and/or may contain a range of other molecules fused, linked, bound or otherwise associated to the protein such as amino acids, lipids, carbohydrates or other peptides, polypeptides or proteins. Reference hereinafter to a "protein" includes a protein comprising a sequence of amino acids as well as a protein associated with other molecules such as amino acids, lipids, carbohydrates or other peptides, polypeptides or proteins.

As hereinbefore defined, the antigen of the present invention may also be a polytope. The subject polytope may be produced by synthetic or recombinant means (for example refer International Patent Publication No. WO 96/03144).

According to this preferred embodiment, there is provided an immunogenic complex comprising a negatively charged organic carrier and a positively charged protein which organic carrier and protein are electrostatically associated.

In this regard, the antigen which is included in the immunogenic complex of the present invention may be, in its initial or natural form, positively charged, negatively charged or of neutral charge. Where an antigen is positively charged, it may nevertheless be weakly positively charged and may therefore require modification to increase its degree of positive charge such that complex formation with the negatively charged organic carrier is better facilitated. For example, wherein an antigen is weakly positively charged, increasing the degree of its positive charge may be achieved by any one of a number of methods known to those skilled in the art including, but not limited to, chemically adding further positive charge to the antigen or recombinantly adding positive charge such as by adding polylysine to the antigen. This is of particular use where the antigen is a protein. Other methods which may be utilised to increase the degree of an antigen's positive charge include, but are not limited to, pH modification, chemical modifications or neutralisation of an antigen's negative charges with positively charged molecules such as arginine. Similarly, where an antigen is neutral or negatively charged, its overall charge can be converted to an overall positive charge by utilising such methodology. Conversion of a negatively charged antigen to express an overall positive charge may be of particular importance where the antigen is a protein, since most proteins are naturally negatively charged.

Once the charge of the antigen of interest is sufficiently positive, it becomes necessary to ensure that precipitation of the positively charged antigen does not occur prior to complex formation with the organic carrier. In this regard, any suitable method for preventing antigen precipitation may be utilised. For example, antigen solubility may be maintained by disrupting the forces that cause antigen aggregation. Disruption of these forces can be

achieved, for example, by incorporating into the antigen solution chaotrophic agents such as urea and guanidine, solvents such as DMSO (dimethyl sulfoxide) and acetonitrile, intermediates such as zwitterions, detergents such as Triton X-100 and CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonate), reducing agents such as DTT (dithiothreitol) and cysteine and chelating agents such as EDTA (ethylene diaminetetraacetic acid). Solubility can also be maintained by altering the pH of the antigen solution or by chemical modification of the antigen to introduce polar or ionic molecules such as by alkylation or acetylation. A gradual or phased removal of these solubilising agents when the antigen has been brought into contact with the "organic carrier" or mild denaturation of the antigen can lead to a controlled precipitation of antigen with concomitant increased association with the organic carrier.

Reference to "organic carrier" should be understood as a reference to any molecule, aggregate or complex of molecules, compound or other entity which, when an antigen is associated with it, facilitates the induction of an immune response, and in particular a cytotoxic T-lymphocyte response, to the antigen. The subject carrier is "organic" and, in this regard, "organic" should be understood as a compound of carbon whether naturally, recombinantly or synthetically obtained or derived. In a particularly preferred embodiment the organic carrier is an adjuvant. By "adjuvant" is meant any organic molecule, aggregate or complex of organic molecules, compound or other entity which functions to stimulate, enhance or otherwise up-regulate any one or more aspects of the immune response. For example, the adjuvant may induce inflammation thereby attracting immune response cells to the site of antigen localisation. Alternatively, the adjuvant may slowly release the antigen thereby providing on-going stimulation of the immune system. Examples of adjuvants suitable for use in the present invention include, but are not limited to, saponin, saponin complexes, any one or more components of the immunostimulating complex of saponin, cholesterol and lipid known as ISCOMATRIX™ (for example the saponin component and/or the phospholipid component), liposomes or oil-in-water emulsions. [The composition and preparation of ISCOMATRIX™ is described in detail in International Patent Application Number PCT/SE86/00480, Australian Patent Numbers 558258 and 632067 and European Patent Publication No. 0 180 564, the disclosures of

which are incorporated herein by reference]. Further examples of adjuvants include, but are not limited to, those detailed in the publication of Cox and Coulter, 1992, 1997 and 1999. It should be understood that the subject organic carrier may be naturally occurring or it may be synthetically or recombinantly derived.

Accordingly, the present invention still more preferably provides an immunogenic complex comprising a negatively charged adjuvant and a positively charged protein which adjuvant and protein are electrostatically associated.

Preferably, said adjuvant comprises saponin or a saponin complex. More preferably, said saponin complex is ISCOMATRIX™.

The organic carrier of the present invention may also be, in its initial or natural form, negatively charged, positively charged or neutral. Increasing the degree of negative charge (for example, where the organic carrier is only weakly negatively charged) or converting a neutral or positively charged organic carrier to a negatively charged organic carrier may also be achieved by any suitable method known to those skilled in the art. For example, where the organic carrier is an oil-in-water emulsion, incorporation of any anionic surfactant with a non-polar tail will impart an overall negative charge to the emulsion due to insertion of the tail of the surfactant into the oil droplet which thereby leaves the negatively charged head group exposed. The negative charge of a saponin complex adjuvant may be increased, for example, by the addition of negatively charged lipid during complex formation.

Examples of detergents which can increase the negative charge of a carrier include, but are not limited to cholic acid, deoxycholic acid, taurocholic acid and taurodeoxycholic acid. Examples of lipids which can increase the negative charge of a carrier include, but are not limited to, phospholipids (preferably phosphatidyl inositol, phosphatidyl serine, phosphatidyl glycerol and phosphatidic acid and most preferably cardiolipin) and bacterial lipids (preferably monophosphoryl lipid A(MPL) and most preferably diphosphoryl lipid A such as OM174 as described in International Patent Publication No. WO 95/14026).

Without limiting the present invention in any way, the inventors have determined that where the subject charged organic carrier and charged antigen are naturally negatively and positively charged, respectively, the object of the invention can be achieved. However, a still more effective immunogenic complex is achieved if the subject naturally negatively charged organic carrier is rendered more negatively charged (preferably by addition of cardiolipin or diphosphory lipid A) and/or the subject naturally positively charged antigen is rendered more positively charged (preferably by addition of a polylysine tail). Preferably, both the naturally negatively charged organic carrier is rendered more negatively charged and the naturally positively charged antigen is rendered more positively charged.

Accordingly, in one preferred embodiment there is provided an immunogenic complex comprising a negatively charged adjuvant and a positively charged protein, wherein said negatively charged adjuvant is a naturally negatively charged adjuvant which has been modified to increase the degree of its negative charge, which adjuvant and protein are electrostatically associated.

In another preferred embodiment there is provided an immunogenic complex comprising a negatively charged adjuvant and a positively charged protein, wherein said positively charged protein is a naturally positively charged protein which has been modified to increase the degree of its positive charge, which adjuvant and protein are electrostatically associated.

In a most preferred embodiment that is provided an immunogenic complex comprising a negatively charged adjuvant and a positively charged protein, wherein said negatively charged adjuvant is a naturally negatively charged adjuvant which has been modified to increase the degree of its negative charge and said positively charged protein is a naturally positively charged protein which has been modified to increase the degree of its positive charge, which adjuvant and protein are electrostatically associated.

Reference to an adjuvant or protein being "naturally" negatively or positively charged, respectively, should be understood as a reference to the charge which the molecule bears upon its creation - whether that be by natural, recombinant or synthetic means. Modification to increase the degree of charge can be achieved by any suitable technique as hereinbefore discussed. Preferably, the subject protein is rendered more positively charged via the addition of a polylysine tail and the subject adjuvant is rendered more negative via the addition of cardiolipin or diphosphoryl lipid A.

Reference to "derivative and equivalents" should be understood as a reference to fragments, parts, portions, chemical equivalents, mutants, homologs and analogs from natural, synthetic or recombinant sources. Where the subject antigen or carrier is a protein, derivatives may be derived from insertion, deletion or substitution of amino acids. Amino acid insertional derivatives include amino and/or carboxylic terminal fusions as well as intrasequence insertions of single or multiple amino acids. Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterised by the removal of one or more amino acids from the sequence. Substitutional amino acid variants are those in which one residue in the sequence has been removed and a different residue inserted in its place. Additions to amino acid sequences include fusions with other peptides, polypeptides or proteins. "Equivalents" can act as a functional analog of the subject carrier or antigen. Chemical equivalents may not necessarily be derived from the subject carrier of antigen but may share certain conformational similarities. Alternatively, chemical equivalents may be designed to mimic certain physiochemical properties of the subject carrier or antigen. Equivalents may be chemically synthesized or may be detected following, for example, natural product screening. Homologs contemplated herein include, but are not limited to, molecules derived from different species.

The present invention is predicated, in part, on the formation of immunogenic complexes via the electrostatic association, preferably, of a negatively charged organic carrier with a positively charged antigen. The administration of such a complex to a subject facilitates

the induction of a significantly better immune response than would be achieved were the adjuvant and antigen administered simultaneously but in a non-associated form. In particular, the administration of an antigen associated with an adjuvant, according to the present invention, facilitates the induction of a cytotoxic T-lymphocyte response to the antigen. However, humoral and other cellular responses can also be enhanced.

Without limiting the present invention to any one theory or mode of action, it is thought that the complexing of the adjuvant with the antigen facilitates co-delivery of the adjuvant and the antigen to the same antigen presenting cell thereby facilitating the induction of immune responses which either would not occur or would not occur as effectively were these molecules not co-delivered. For example, the induction of some CD8+ cytotoxic T-lymphyocyte responses are thought to occur where the adjuvant induces endosomal escape of the antigen in the antigen presenting cell. This necessarily requires co-delivery of the antigen and the adjuvant to the antigen presenting cell.

A further aspect of the present invention therefore relates to the use of the invention to induce an immune response in a mammal including, but not limited to, a humoral and/or cell mediated immune response.

Accordingly, another aspect of the present invention relates to a vaccine composition comprising as the active component an immunogenic complex comprising a charged organic carrier and a charged antigen which organic carrier and antigen are electrostatically associated together with one or more pharmaceutically acceptable carriers and/or diluent.

Preferably, said organic carrier is an adjuvant, and even more preferably a saponin or a saponin complex. Preferably said saponin complex is ISCOMATRIX™.

Still more preferably, said antigen is a protein.

Preferably said organic carrier is negatively charged and said antigen is positively charged.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion or may be in the form of a cream or other form suitable for topical application. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The organic carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of superfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filter sterilisation. Generally, dispersions are prepared by incorporating the various sterilised active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

When the active ingredients are suitably protected they may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in such therapeutically useful compositions is such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 0.1 μg and 2000 mg of active compound.

The tablets, troches, pills, capsules and the like may also contain the components as listed hereafter: a binder such as gum, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound(s) may be incorporated into sustained-release preparations and formulations.

Without limiting the operation of the present invention in any way, the co-delivery of the immunogenic complex of the present invention is particularly useful for inducing an immune response and, in particular, a cytotoxic T-lymphocyte response to an antigen said immune response may be a specific (T cell and/or B cell) and/or non-specific immune response.

Accordingly, still another aspect of the present invention relates to a method of eliciting, inducing or otherwise facilitating, in a mammal, an immune response to an antigen said method comprising administering to said mammal an effective amount of an immunogenic complex or a vaccine composition as hereinbefore described.

Preferably said immune response is a cytotoxic T-lymphocyte response.

It should be understood that the subject cytotoxic lymphocyte response may occur either in isolation or together with a helper T cell response, a humoral response or other specific or non-specific immune response.

A further aspect of the present invention relates to the use of the immunogenic complex of the invention in relation to the therapeutic and/or prophylactic treatment of disease conditions. Examples of disease conditions which can be treated in accordance with the method of the present invention include, but are not limited to, any disease condition which results from a microbial infection or a cancer. Examples include HIV, Hepatitis B, Hepatitis C, melanoma, prostate cancer, breast cancer, tuberculosis and parasitic conditions.

Accordingly, yet another aspect of the present invention relates to a method of treating a disease condition in a mammal said method comprising administering to said mammal an effective amount of an immunogenic complex or a vaccine composition as hereinbefore described wherein administering said composition elicits, induces or otherwise facilitates an immune response which inhibits, halts, delays or prevents the onset or progression of the disease condition.

An "effective amount" means an amount necessary at least partly to attain the desired immune response, or to delay the onset or inhibit progression or halt altogether, the onset or progression of a particular condition being treated. This amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of individual to be treated, the capacity of the individual's immune system to synthesize antibodies, the degree of protection desired, the formulation of the vaccine, the assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

The term "mammal" includes humans, primates, livestock animals (eg. horses, cattle, sheep, pigs, donkeys), laboratory test animals (eg. mice, rats, rabbits, guinea pigs), companion animals (eg. dogs, cats) and captive wild animals (eg. kangaroos, deer, foxes). Preferably, the mammal is a human or laboratory test animal. Even more preferably, the mammal is a human.

The mammal undergoing treatment may be human or an animal in need of therapeutic or prophylactic treatment of a disease condition or a potential disease condition.

In yet another aspect the present invention relates to the use an immunogenic complex or vaccine composition as hereinbefore defined in the manufacture of a medicament for inhibiting, halting, delaying or preventing the onset or progression of a disease condition.

Yet another aspect of the present invention relates to an agent for use in inhibiting, halting, delaying or preventing the onset or progression of a disease condition. Said agent comprising an immunogenic complex or vaccine composition as hereinbefore defined.

Further features of the present invention are more fully described in the following non-limiting Examples.

Reference to "ISCOPREP™ 703" should be understood as a reference to a saponin preparation comprising from 50-90% by weight of Fraction A of Quil A and 50% to 10% by weight of Fraction C of Quil A. Fractions A and C are prepared from the lipophilic fraction of Quil A. Fractions "A" and "C", their method of preparation and the method of preparing 703 are detailed in International Patent Publication No. WO96/11711, which is incorporated herein by reference.

EXAMPLE 1

PREPARATION OF STANDARD AND MODIFIED ISCOMATRIX™

ISCOMATRIX™ (Immunostimulating complex without antigen) was prepared essentially by the method of Morein *et al.* (1989). Briefly, to 1.76 ml PBS pH 7.2 was added 0.16 ml of a solution containing 10 mg/ml tritiated (^{3}H) cholesterol and 10 mg/ml lipid in 20% MEGA-10 detergent (w/v) then 0.08 ml of a solution containing 100 mg/ml ISCOPREP™ 703 in PBS. The solution was held at 25°C for 1 hour with gentle mixing. During subsequent dialysis against PBS/azide, ISCOMATRIX™ containing cholesterol, DPPC and ISCOPREP™ was formed. All the ISCOMATRIX™ formulations were of typical appearance by electron microscopy.

Lipids:

Standard DPPC	dipalmitoylphosphatidylcholine
CDL modified	cardiolipin
DPL modified	diphosphoryl lipid A
MPL modified	monophosphoryl lipid A
DPA modified	phosphatidic acid
DPPG modified	dipalmitoylphosphatidyl glycerol

After formulation, preparations were purified on a sucrose gradient (10 to 50% w/v) and fractions analysed for lipid and cholesterol. Cholesterol was detected by ^{3}H cpm of 100μl sample in 1ml scintillant and lipid was detected using diphenylhexatriene (DPH) which

fluoresces when associated with lipid. Briefly, DPH was dissolved at 1mg/ml in acetone then diluted 1 in 50 in PBS pH7.2, then 50μl mixed with 50μl of each fraction in a microtitre plate. Following incubation for 150 mins at 20-25^{0}C the plate was read in a fluorometer using excitation 355nm and emission 460nm. The DPH and ^{3}H peaks coincided for all formulations and the gradient profiles of the modified formulations were similar to the standard formulation indicating incorporation of the lipid into the ISCOMATRIX™ (Figure 1).

EXAMPLE 2

PREPARATION OF ANTIGEN ASSOCIATED ISCOMATRIX™ WITH A NATURALLY POSITIVELY CHARGED PROTEIN: *H.pylori* family E protein (HpE)

The HpE protein has a pI of 9.24 making it a postively charged protein at pH8. Solubility of the HpE was maintained using 0.5M Tris, 0.5M NaCl, 0.1% 1,2-Diheptanoyl-sn-Glycero-3-phosphocholine (DHPC) pH8. The HpE associated ISCOMATRIX™ formulations were prepared by mixing at a 1:5 ratio of protein to ISCOPREP™ as ISCOMATRIX™ for 60 minutes at 20-25°C. The ISCOMATRIX™ formulations used were DPPC, CDL, DPL and DPPG.

After formulation, preparations were purified on a sucrose gradient (10 to 50% sucrose w/v) and fractions analysed for HpE, association between HpE and ISCOMATRIX™ and ISCOMATRIX™ (Figure 2). HpE was detected by adsorbing fractions diluted 1 in 10 in PBS to wells of an EIA plate then detecting with a Horse radish peroxidase (HRP) conjugated monoclonal antibody to HpE. Association was determined by EIA using a monoclonal antibody to HpE to capture and a HRP conjugated monoclonal antibody to ISCOPREP™ to detect. ISCOMATRIX™ was determined by detecting ^{3}H cholesterol.

The HpE protein, when not mixed with ISCOMATRIX™, was found in fractions 3-10 by EIA. (Figure 2E). When mixed with DPPC ISCOMATRIX™ the HpE was found predominantly in fractions 2-8 but some was found in fractions 12-20 coinciding with the

ISCOMATRIX™ and association peaks which indicates that association occurred (Figure 2A). When mixed with CDL or DPL ISCOMATRIX™ the HpE was found predominantly in fractions 7-16 coinciding with the ISCOMATRIX™ and association peaks which indicates that almost complete association occurred (Figure 2B&C). There was very little, if any, free HpE found in fractions 2-8. When mixed with DPPG ISCOMATRIX™ the results were similar to the DPPC ISCOMATRIX™ (Figure 2D).

These results indicate that DPPG and standard DPPC ISCOMATRIX™ can associate weakly with antigens that are positively charged and the capacity to associate can be substantially increased by using CDL or DPL ISCOMATRIX™.

EXAMPLE 3
PREPARATION OF ANTIGEN ASSOCIATED ISCOMATRIX™ WITH A NATURALLY POSITIVELY CHARGED PROTEIN: NY-ESO-1 (ESO)

The ESO protein has a pI of 9.1 making it a positively charged protein at pH7. Solubility of the ESO was maintained using 8M Urea, 50mM Tris, 50mM $NaH_2PO_4.2H_2O$, 0.15M NaCl pH7. The ESO associated ISCOMATRIX™ formulations were prepared by mixing at a 1:5 ratio of protein to ISCOPREP™ as ISCOMATRIX™ for 60 minutes at 20-25°C. The ISCOMATRIX™ formulations used were DPPC and DPL.

After formulation, preparations were purified on a sucrose gradient (10 to 50% sucrose w/v) and fractions analysed for ESO, association between ESO and ISCOMATRIX™ and ISCOMATRIX™ (Figure 3). ESO was detected by adsorbing fractions diluted 1 in 10 in PBS to wells of a EIA plate then detecting with a HRP conjugated monoclonal antibody to ESO. Association was determined by EIA using a monoclonal antibody to ESO to capture and a HRP conjugated monoclonal antibody to ISCOPREP™ to detect. ISCOMATRIX™ was determined by detecting ^3H cholesterol.

The ESO protein, when not mixed with ISCOMATRIX™, was found in fractions 1-6 by EIA. (Figure 3C). When mixed with standard DPPC ISCOMATRIX™ ESO was found in fractions 1-6 and 12-16 (Figure 3A). The presence in fractions 12-16 coincided with the ISCOMATRIX™ and association peaks indicating there was association but a large proportion of the ESO was not associated as indicated by the presence in fractions 1-6. When mixed with DPL ISCOMATRIX™ the ESO was found predominantly in fractions 12 to 16 coinciding with the ISCOMATRIX™ and association peaks which indicates that association occurred (Figure 3B).

These results show there was some association of a positively charged protein with standard DPPC ISCOMATRIX™ but the capacity to associate was substantially increased by use of DPL ISCOMATRIX™.

EXAMPLE 4

IMMUNISATION OF MICE WITH ESO ASSOCIATED STANDARD ISCOMATRIX™

Antibody Responses:

Ten BALB/c mice were immunised, on days 0 and 28, subcutaneously in the scruff of the neck with 0.1ml of ESO containing 5μg protein or ESO associated ISCOMATRIX™ containing 5μg protein and 5μg ISCOPREP™. The mice were bled on day 35 and the sera analysed for antibodies to ESO by indirect EIA. Briefly, ESO was adsorbed to a microtitre plate in PBS pH7.2 then the plate blocked with a 0.1% casein solution and dried. Dilutions of sera were incubated for 1 hour at 20-25°C then the plates washed. HRP conjugated goat anti mouse IgG, IgG_1 or IgG_{2a} was added and plates incubated for 1 hour at 20-25°C then washed. TMB substrate was added and incubated for 10 mins at 20-25°C followed by addition of 0.5M H_2SO_4 to stop the reaction. Plates were read at OD450nm and end point titres calculated.

There was a greater than 20 fold increase in the IgG and IgG_1 responses to ESO when associated with ISCOMATRIX™ and a thousand fold increase in IgG_{2a} titre (Figure 4).

Cytotoxic T Lymphocyte (CTL) Responses:

Five HLA A2 transgenic HHD mice were immunised subcutaneously at the base of the tail with 0.1ml of ESO containing 5μg protein or ESO associated ISCOMATRIX™ containing 5μg protein and 5μg ISCOPREP™. After 14 days splenocytes were harvested and $5X10^6$ cells restimulated in 24-well plates with EL4HHD cells sensitised with ESO peptide (10μg/ml for 1 hour 37°C), irradiated and washed twice. Cells were cultured in RPMI media supplemented with 10% foetal calf serum, 2mM glutamine, $5X10^{-5}$ Mβ-mercaptoethanol, 100μg/ml strepomycin and 100IU/ml pencillin and incubated at 37°C for 6 days in 5%CO_2. On day 4 1ml of medium was added containing 5U/ml recombinant human IL-2. On day 6 the cultures were used as effectors in standard 6 hour ^{51}Cr release assays against EL4HHD cells sensitised as for restimulation.

CTL were not detected in mice immunised with ESO alone but when associated with ISCOMATRIX™, CTL was detected in all mice (Figure 5).

These results indicate that association is required for optimal induction of cellular immune responses.

EXAMPLE 5

PREPARATION OF ANTIGEN ASSOCIATED ISCOMATRIX™ WITH A NATURALLY NEGATIVELY CHARGED PROTEIN: HPV E6E7 (E6E7)

The E6E7 protein has a pI of 5.9 making it a negatively charged protein at pH6.9. Solubility of the E6E7 was maintained using 8M Urea, 50mM Tris, 50mM $NaH_2PO_4.2H_2O$, 150mM NaCl pH6.9. The E6E7 associated ISCOMATRIX™ formulations were prepared by mixing at a 1:5 ratio of protein to ISCOPREP™ as

ISCOMATRIX™ for 60 minutes at 20-25°C. The ISCOMATRIX™ formulations used were DPPC, CDL, DPL, MPL, DPA and DPPG.

After formulation, preparations were purified on a sucrose gradient (10 to 50% sucrose w/v) and fractions analysed for E6E7, association between E6E7 and ISCOMATRIX™ and ISCOMATRIX™ (Figure 8). E6E7 was detected by EIA using two non-competing monoclonal antibodies to E7. Association was determined by EIA using a monoclonal antibody to E7 to capture and a HRP conjugated monoclonal antibody to ISCOPREP™ 703 to detect. ISCOMATRIX™ was determined by detection of ^{3}H cholesterol.

The E6E7 protein alone was found in fractions 10-22 by EIA (Figure 6G). When mixed with standard DPPC ISCOMATRIX™ most of the E6E7 found was in fractions 14-20 with little association detected (Figure 6A). When mixed with CDL, DPL and DPA ISCOMATRIX™ the E6E7 was found predominantly in fractions which coincided with the association and the ISCOMATRIX™ peaks which indicated that almost complete association occurred (Figure 6B,C, E). When mixed with MPL and DPPG ISCOMATRIX™ the protein was found in fractions 9-14 coinciding with the association and ISCOMATRIX™ peaks indicating association but a significant amount found not associated in fractions 17-22 (Figure 6D,F).

These results indicate that a negatively charged protein binds poorly to standard DPPC ISCOMATRIX™ and the capacity to associate increases by using CDL, DPL, MPL, DPA or DPPG to varying degrees.

EXAMPLE 6

IMMUNISATION OF MICE WITH E6E7 ASSOCIATED STANDARD AND MODIFIED ISCOMATRIX™

Three C57BL/6 mice were immunised, on day 0 and day 21, subcutaneously with 0.1ml of E6E7 associated ISCOMATRIX™ containing 10μg protein and 6μg ISCOPREP™. After 7 days splenocytes were harvested and $20X10^{6}$ cells restimulated in 8mL in a T25 tissue

culture flask with E7 transfected EL4 cells (C2) mytomycin-C treated and washed three times. Cells were cultured in RPMI media supplemented with 10% foetal calf serum, 2mM glutamine, $5.5X10^{-5}$ Mβ-mercaptoethanol, 50μg/ml gentamicin and incubated at 37°C for 5 days in 5%CO_2. On day 6 the cultures were used as effectors in a standard 4 hour ^{51}Cr release assays against C2 cells.

The E6E7 associated DPL ISCOMATRIX™ induced a CTL response in 2 out of 3 mice (Figure 7A). The E6E7 associated with standard DPPC ISCOMATRIX™ failed to induce a CTL response in any mice (Figure 7B). The negative mouse in the DPL ISCOMATRIX™ group had insufficient cells for optimal readout and would not comply with criteria for a valid response. All other mice fulfilled criteria for valid responses.

These results show that the greater the association the better the CTL response.

EXAMPLE 7

PREPARATION OF ANTIGEN ASSOCIATED ISCOMATRIX™ WITH A NATURALLY NEGATIVELY CHARGED PROTEIN: *H.pylori* family C protein (HpC)

The HpC protein has a pI of 5.05 making it negatively charged at pH7.2. The protein was soluble in PBS pH7.2. The HpC associated ISCOMATRIX™ formulations were prepared by mixing at a 1:5 ratio of protein to ISCOPREP™ as ISCOMATRIX™ for 60 minutes at 20-25°C. The ISCOMATRIX™ formulations used were DPPC and DPL.

After formulation, preparations were purified on a sucrose gradient (10 to 50% sucrose w/v) and fractions analysed for HpC, association between HpC and ISCOMATRIX™ and ISCOMATRIX™ (Figure 8). HpC was detected by adsorbing fractions diluted 1 in 10 in PBS to wells of an EIA plate then detecting with a HRP conjugated monoclonal antibody to HpC. Association was determined by EIA using a monoclonal antibody to HpC to capture and a HRP conjugated monoclonal antibody to ISCOPREP™ to detect.

ISCOMATRIX™ was determined by either detection of ^{3}H cholesterol or DPH as described in example 1.

HpC alone was found in fractions 1-5 and when mixed with standard DPPC ISCOMATRIX™ the HpC was found predominantly in fractions 1-5 and not associated. When mixed with DPL ISCOMATRIX™ a significant proportion of the HpC was found in fractions 11-17 coinciding with the ISCOMATRIX™ and association peaks indicating association.

These results indicate that a negatively charged protein binds poorly to standard DPPC ISCOMATRIX™ and the capacity to associate increases by using DPL ISCOMATRIX™.

EXAMPLE 8

PREPARATION OF ANTIGEN ASSOCIATED ISCOMATRIX™ WITH A NATURALLY POSITIVELY CHARGED PROTEIN UTILISING pH TO GIVE A POSITIVE CHARGE: E6E7.

The E6E7 protein has a pI of 5.9 making it a negatively charged protein at pH7.2. It contains a hexa histidine sequence at the N terminus which will be positively charged at pH6. Solubility of the E6E7 was maintained using 8M urea, 50mM Bis Tris, 0.15M NaCl pH6. The E6E7 associated ISCOMATRIX™ formulation was prepared by mixing equal mass of E6E7 with ISCOPREP™ as ISCOMATRIX™ for 60 minutes at 20-25°C, dialysing against 50mM Bis Tris, 0.15M NaCl pH6 to remove the urea then centrifugation at 10,000 g for 5 mins to remove any precipitate.

After formulation, preparations were purified on a sucrose gradient (50 to 10% sucrose w/v) and fractions analysed for protein, association between E6E7 and ISCOMATRIX™ and ISCOMATRIX™ (Figure 9). Protein was detected using a sandwich EIA for E7. Association was determined by EIA using a monoclonal antibody to E7 to capture and a HRP conjugated monoclonal antibody to ISCOPREP™ to detect. ISCOMATRIX™ was determined by detection of ^{3}H cholesterol or DPH as described in example 1.

E6E7 was found in fractions 10-22 when run alone (Figure 9C). When mixed with DPPC ISCOMATRIX™ at pH7.2 the E6E7 was predominantly found in fractions 16-22 with little evidence of association (Figure 9B). When mixed with standard DPPC ISCOMATRIX™ at pH6 the E6E7 was predominantly found in fractions 12-16 coinciding with the ISCOMATRIX™ and association peaks which indicates association(Figure 9A).

These results show that pH can be used to increase the capacity of standard DPPC ISCOMATRIX™ to associate with naturally negatively charged proteins.

EXAMPLE 9

IMMUNISATION OF MICE WITH pH MODIFIED E6E7 ASSOCIATED DPPC ISCOMATRIX™

Six C57BL/6 mice were immunized, on days 0 and 21, subcutaneously in the scruff of the neck with 0.1 ml of E6E7 associated ISCOMATRIX™ containing 6μg ISCOPREP™ and 6μg E6E7.

Antibody Responses:

Mice were bled on day 26 and sera analysed for antibodies to E7 by indirect EIA. Purified GSTE7 was adsorbed to a microtitre plate in 0.1M Carbonate pH9.6 then the plate blocked with a 0.1% casein solution and dried. Dilutions of sera were incubated for 1 hour at 20-25°C then the plates washed. HRP conjugated goat anti mouse IgG was added and plates incubated for 1 hour at 20-25°C then washed. TMB substrate was added and incubated for 10 mins at 20-25°C followed by addition of 0.5M H_2SO_4 to stop the reaction. Plates were read at OD450nm and end point titres calculated.

The E6E7 associated ISCOMATRIX™ group had a GMT of 949. Typically E6E7 with $Al(OH)_3$ gives GMT of approximately 100.

Cytokine Responses:

On day 27 splenocytes from each of 3 mice were harvested and pooled and $2.5X10^6$ cells restimulated in 48-well plates with GSTE7 at 1 and 5 μg with ConA and RPMI as controls. Cells were cultured in RPMI media supplemented with 10% foetal calf serum, 2mM glutamine, $5X10^{-5}$ Mβ-mercaptoethanol, 100μg/ml streptomycin and 100IU/ml pencillin and incubated at 37°C for 2 days in 5%CO_2. The supernatant was harvested and γIFN and IL5 detected by EIA using reagents from Endogen.

The E6E7 associated ISCOMATRIX™ induced up to 7.4 ng/ml γIFN and 140 pg/ml IL5 (Table 2). Typically E6E7 with $Al(OH)_3$ induces no detectable γIFN (<30 pg/ml) or IL5 (<4 pg/ml).

These results show that pH modified E6E7 associated ISCOMATRIX™ were immunogenic in mice and induced a Th1 type response.

EXAMPLE 10

PREPARATION OF CHELATING (CHL) ISCOMATRIX™

CHL ISCOMATRIX™ was prepared by the method of Macfarlan and Malliaros, (1998) International Patent Publication No. WO 98/36772). Briefly, to 1.6 ml 50mM Tris, 150mM NaCl, 0.6mM $CuCl_2$ pH 7.2 (Buffer A) was added 0.2 ml of a solution containing 10 mg/ml cholesterol, 9 mg/ml DPPC, 1.074 mg/ml dipalmitoyl-rac-glycerol-3(8-(3,6-dioxy) octyl-1-amino-N,N-diacetic acid) (DPIDA) in 20% MEGA-10 detergent (w/v) then 0.2 ml of a solution containing 50 mg/ml ISCOPREP™ 703 in Buffer A. The solution was held at 25°C for 90 mins with gentle mixing. Dialysis was then performed firstly against Buffer A overnight with 2 changes of buffer then against 50mMTris, 50mM $NaH_2PO_4.2H_2O$, 150mM NaCl pH6.9 for 2 days with two changes of buffer. During dialysis CHL ISCOMATRIX™ containing cholesterol, DPPC, DPIDA and ISCOPREP™ was formed. The CHL ISCOMATRIX™ formulation was of typical appearance by electron microscopy.

EXAMPLE 11

GENERATION, EXPRESSION AND PURIFICATION OF HEXAHISTIDINE (6H) ± HEXALYSINE (6K) HpC.

The HpC protein has a pI of 5.05 making it negatively charged at pH7.2. Addition of 6K would change the pI to 7.68 and give a positively charged tail. Two clones were constructed to give HpC plus 6H, for purification, and with and without 6K. CSL 694 DNA (HpC13 with a C-terminal 6H in the vector pGexStop as described in Edwards *et al.* 1998) was used as the template for PCR amplification to generate a C-terminal 6K. The PCR product was cloned into the EcoRI-BglII sites of the expression vector pGexStopIV, creating tandem C-terminal 6K followed by 6H tags. This was generated in the *E. coli* strain ER1793 and designated CSL 1424.

One litre cultures were induced at A_{600}=2 with 0.5mM IPTG and harvested 5 hours post induction. Soluble recombinant protein was purified utilising the C-terminal 6H tag for metal (nickel) affinity chromatography. Eluted protein was dialysed against PBS.

EXAMPLE 12

PREPARATION OF ANTIGEN ASSOCIATED ISCOMATRIX™ WITH 6H AND 6K TAGS : HpC.

The HpC protein with 6H has a pI of 5.85 making it negatively charged at pH7.2. Addition of a 6K to this protein gives a pI of 7.68 making it positively charged at pH7.2. Both forms of the protein were soluble in PBS pH7.2. The HpC associated ISCOMATRIX™ formulations were prepared by mixing at a 1:5 ratio of protein to ISCOPREP™ as ISCOMATRIX™ for 60 minutes at 20-25°C. The ISCOMATRIX™ formulations used were DPPC and CHL. CHL ISCOMATRIX™ technology was used as a standard method for associating 6H proteins with ISCOMATRIX™.

After formulation, preparations were purified on a sucrose gradient (10 to 50% sucrose w/v) and fractions analysed for HpC, association between HpC and ISCOMATRIX™ and ISCOMATRIX™ (Figure 10). HpC was detected by adsorbing fractions diluted 1 in 10 in PBS to wells of an EIA plate then detecting with a HRP conjugated monoclonal antibody to HpC. Association was determined by EIA using a monoclonal antibody to HpC to capture and a HRP conjugated monoclonal antibody to ISCOPREP™ to detect. ISCOMATRIX™ was determined by detection of DPH as described in example 1.

When mixed with standard DPPC ISCOMATRIX™ the 6H-HpC was found in fractions 1-6 and with little evidence of association (Figure 10C). When 6K6H-HpC was mixed with DPPC ISCOMATRIX™ a significant amount of HpC was in fractions 7-11 coinciding with the association and ISCOMATRIX™ peaks indicating association (Figure 10A). When 6H-HpC was mixed with CHL ISCOMATRIX™ most of the HpC was in fractions 7-14 coinciding with the ISCOMATRIX™ and association peaks indicating association (Figure 10B).

These results show that addition of a 6K to a negatively charged protein increased its capacity to associate with standard DPPC ISCOMATRIX™ and the association achieved was comparable to that using 6H with CHL ISCOMATRIX™.

EXAMPLE 13

PREPARATION OF SYNTHETIC POLYTOPE ISCOM™ AND ASSOCIATED ISCOMATRIX™ WITH PALMITIC ACID (PAL), 6H, 6K AND NO FORMULATION TAGS.

The polytopes were synthesised and purifed by Chiron Technologies on Multipin (TM) crowns, as described by Valerio *et al.*, using the Fmoc alpha-amino protection scheme for the amino acids. After sidechain deprotection and cleavage in a trifluoracetic acid/scavenger solution, peptides were precipitated with ether and dried. The redissolved peptide was purified by preparative reverse phase HPLC using elution with a gradient of

acetonitrile. Fractions containing material of the correct molecular mass, as determined by ion spray mass spectrometry, were pooled and dried.

The polytope was as follows:

Tag-YPHFMPTNLRPQASGVYMTYQRTRALVSYIPSAEKI-OH (<400>3) containing four known BALB/c restricted epitopes, YPHFMPTNL (<400>4), RPQASGVYM (<400>5), TYQRTRALV (<400>6) and SYIPSAEKI (<400>7). The tags used were PAL, 6H, 6K or H (No tag).

For the PAL polytope association was achieved by incorporation into ISCOM™ (Immunostimulating complex) according to the method of Morein *et al.* (1989). Briefly, to 4 mg of polytope solubilised in 1.76 ml 10% MEGA-10 detergent (w/v), 50% Acetonitrile in PBS was added 0.16 ml of a solution containing 10 mg/ml cholesterol and 10 mg/ml DPPC in 20% MEGA-10 detergent (w/v) then 0.08 ml of a solution containing 100 mg/ml ISCOPREP™ 703 in PBS. The solution was held at 25°C for 1 hour with gentle mixing. During subsequent dialysis against PBS/azide ISCOMs™ containing palmityfied polytope, cholesterol, DPPC and ISCOPREP™ were formed. These ISCOMs™ were of typical appearance by electron microscopy.

The 6H polytope was solubilised in 8M urea then mixed with CHL ISCOMATRIX™ and the 6K and no tag polytopes were solubilised in PBS then mixed with standard DPPC ISCOMATRIX™. All formulations were prepared at a ratio of 1:8 protein to ISCOPREP™ as ISCOMATRIX™ and incubated for 60 mins at 20-25°C.

The preparations were purified on a sucrose gradient (10 to 50% sucrose w/v) and fractions analysed for protein and ISCOMATRIX™. Protein was detected using CBQCA (<400>8) from Molecular Probes according to the manufacturers instructions or by Coomassie according to the method of Bradford (1976). Briefly 100µl of each fraction was added to a microplate followed by addition of 100µl Coomassie reagent then the plate read at 595nm. ISCOMATRIX™ was detected by DPH as described in example 1.

The polytope alone was found in fractions 1-5 (Figure 11E). The protein, as detected by CBQCA (<400>8), in the PAL polytope ISCOM™ was found predominantly in fractions 11-13 coinciding with the ISCOMATRIX™ peak indicating incorporation (Figure 11A). The protein, as detected by Coomassie, in the 6K polytope associated ISCOMATRIX™ was found predominantly in fractions 1-5 and was probably not associated with ISCOMATRIX™ (Figure 11C). A significant proportion of polytope was found in fractions 12-14 coinciding with the ISCOMATRIX™ peak indicating association. The protein, as detected by Coomassie, in the 6H polytope associated CHL ISCOMATRIX™ was found predominantly in fractions 4-10 coinciding with the ISCOMATRIX™ peak indicating association (Figure 11B). There was a significant proportion of 6H polytope found in fractions 1-3 which was probably not associated. The protein, as detected by Coomassie, in the no tag associated ISCOMATRIX™ was almost all found in fractions 1-5 and probably not associated with ISCOMATRIX™.

These results show that a tag was required for association of the polytope tested with ISCOMATRIX™ and that 6Kpolytope association with standard DPPC ISCOMATRIX™ was comparable to incorporation of hydrophobic PAL polytope into ISCOMs™ but not as good as 6H polytope association with CHL ISCOMATRIX™.

EXAMPLE 14

IMMUNISATION OF MICE WITH SYNTHETIC POLYTOPE ISCOM™ AND ASSOCIATED ISCOMATRIX™ FORMULATIONS

Three BALB/c mice were immunized subcutaneously at the base of the tail with 0.1 ml of polytope ISCOM™ or associated ISCOMATRIX™ containing 6μg ISCOPREP™ and between 3.5μg and 5μg protien.

CTL assays were performed according to the method of Elliott *et al.* (1999). Briefly, splenocytes from each spleen were removed on day 14 and cultured in 1 ml medium at $5X10^6$ cell/ml, in a 24 well plate, together with 1 μg/ml of the individual peptides (4

peptides/spleen) in a humidifed incubator at 37°C. On day 3, 1 ml of fresh media was added and then further in vitro restimulation performed on day 7 by adding irradiated (800 rad) peptide sensitised (10 μg/ml, 1 hr 37°C, 2 washes) P815 cells at a responder to stimulator ratio of 20:1 to $2X10^6$ effectors/well. The procedure was repeated twice more at 7 day intervals and the bulk cultures were used as effectors 6 days later in a standard 6 hr chromium release assay. Medium contained RPMI 1640 supplemented with 10% FCS (QIMR), $5X10^{-5}$ M 2-mercaptoethanol, 2mM glutamine and pen/strep antibiotics. Target cells were ^{51}Cr labelled peptide sensitised and unsensitised (control) P815 cells. The ratio of effector:target was 50, 10 and 2 to 1. The assays were performed in 96 well round bottom plates in duplicate.

The PAL polytope ISCOM™ induced CTL responses against all 4 epitopes with 3/3 mice for TYQ, 1/3 for SYI, 2/3 for YPH and 2/3 for RPQ (Figure 12A). The 6H polytope associated CHL ISCOMATRIX™ induced CTL responses against 3/3 mice for all 4 epitopes (Figure 12B). The 6K polytope associated DPPC ISCOMATRIX™ induced CTL responses against all 4 epitopes with 3/3 for TYQ, YPH and RPQ and 2/3 for SYI (Figure 12C). The no tag polytope associated DPPC ISCOMATRIX™ induced a weak CTL response in 2/3 mice for RPQ but there was no CTL response detected to any of the other epitopes (Figure 12D). The SYI sequence is known to be a weak epitope and this was the case for all formulations.

These results show that association of polytope with the ISCOM™ or ISCOMATRIX™ was required for optimal CTL induction and that association using 6K was as effective as 6H with CHL ISCOMATRIX™ or classical incorporation of hydrophobic proteins (PAL polytope ISCOM™).

EXAMPLE 15

GENERATION, EXPRESSION AND PURIFICATION OF RECOMBINANT (r) 6H ± 6K POLYTOPE.

Pstmpdv DNA(supplied by QIMR) was used as the template for PCR amplification of the murine polytope, YPHFMPTNLTSSGPSNTPPEIFAPGNYPALSYIPSAEKIEEGAIVGEI RPQASGVYM (<400>9), to enable generation with and without a C-terminal 6K (CSL 1430 and 1426 respectively). PCR products were cloned into the BamHI-XhoI sites of the expression vector pET24b (Novagen) generating an N-terminal T7-tag (for identification) and tandem C-terminal 6K followed by 6H (for purification).

Clones were generated in the *E. coli* strain ER1793 and subsequently transformed into the expression strain BL21(DE3). One litre cultures were induced at A_{600}=2 with 0.5mM IPTG and harvested 4 hours post induction. Soluble recombinant protein was purified utilising the C-terminal 6H tag for metal (nickel) affinity chromatography. Eluted protein was dialysed against PBS.

EXAMPLE 16

PREPARATION OF rPOLYTOPE ASSOCIATED ISCOMATRIX™ 6H AND 6K.

The murine polytope with 6H has a pI of 5.85 making it negatively charged at pH7.2. Addition of a 6K to this gives a pI of 7.68 making it positively charged at pH7.2. Both forms of the protein were soluble in PBS pH7.2. The polytope associated ISCOMATRIX™ formulations were prepared by mixing at a 1:5 ratio of protein to ISCOPREP™ as ISCOMATRIX™ for 60 minutes at 20-25°C. The ISCOMATRIX™ formulations used were DPPC, CDL, DPL and CHL. Formulation at pH4.3 to be below the pI of glutamic acid (E) was investigated as there were a number of E's in the sequence which could potentially interfere with association.

After formulation, preparations were purified on a sucrose gradient (10 to 50% sucrose w/v) and fractions analysed for protein and ISCOMATRIX™ (Figure 13). Protein was

detected by adsorbing fractions diluted 1 in 10 in PBS to wells of an EIA plate then detecting with a HRP conjugated monoclonal antibody to 6H. ISCOMATRIX™ was determined by of detection ^{3}H cholesterol or DPH as described in example 1.

The protein in the r6H6K polytope alone was found in fractions 1-6 (Figure 13K). The protein in the r6K6H polytope mixed with standard DPPC ISCOMATRIX™ at pH7 was found predominantly in fractions 1-9 with little evidence of association (Figure 13 A). The protein in the r6K6H polytope mixed with CDL and DPL ISCOMATRIX™ at pH 7 was found predominantly in fractions coinciding with ISCOMATRIX™ indicating association (Figure 13 C, E). There was a significant proportion of protein found in fractions 1-3 and 1-6, for CDL and DPL respectively, and probably not associated. The protein in the r6K6H polytope mixed with standard DPPC ISCOMATRIX™ at pH4.3 was found predominantly in fractions 1-9 with some evidence of association in the coinciding ISCOMATRIX™ peak in fractions 10-12 (Figure 13 B). The protein in the r6K6H polytope mixed with CDL and DPL ISCOMATRIX™ at pH4.3 was almost all found in fractions coinciding with ISCOMATRIX™ indicating almost complete association (Figure 13 D, F). The r6H polytope mixed with DPPC ISCOMATRIX™ was found predominanlty in fractions 1-7 with little evidence of association. The r6H polytope mixed with CHL ISCOMATRIX™ showed similar patterns of association for standard DPPC and CDL ISCOMATRIX™ at both pH7 and pH4.3. The protein was found in about equal amounts in fractions 1-4 non-associated and 5-10 coniciding with the ISCOMATRIX™ peak indicating association (Figure 13 H, I, J).

These results show that the rpolytope used here would not associate with standard ISCOMATRIX™ even with the addition of 6K. Association could be achieved using modified ISCOMATRIX™ and the capacity to associate with these formulations was increased by utilising low pH. The combination of modified ISCOMATRIX™ and low pH resulted in as good as, or better, association than with 6H CHL ISCOMATRIX™ which was not increased by use of modified ISCOMATRIX™ or low pH.

EXAMPLE 17

IMMUNISATION OF MICE WITH rPOLYTOPE ASSOCIATED ISCOMATRIX™ FORMULATIONS

Three BALB/c mice were immunized subcutaneously at the base of the tail with 0.1 ml of associated ISCOMATRIX™ containing 6μg ISCOPREP™ 703 and between 3.5μg and 5μg protein.

CTL assays were performed according to the method of Elliott *et al.* (1999). Briefly, splenocytes from each spleen were removed on day 14 and cultured in 1 ml medium at $5X10^6$ cell/ml, in a24 well plate, together with 1μg/ml of the individual peptides (4 peptides/spleen) in a humidifed incubator at 37°C. On day 3, 1 ml of fresh media was added and then further in vitro restimulation performed on day 7 by adding irradiated (800 rad) peptide sensitised (10μg/ml, 1 hr 37°C, 2 washes) P815 cells at a responder to stimulator ratio of 20:1 to $2X10^6$ effectors/well. The procedure was repeated twice more at 7 day intervals and the bulk cultures were used as effectors 6 days later in a standard 6 hr chromium release assay. Medium contained RPMI 1640 supplemented with 10% FCS (QIMR), $5X10^{-5}$ M 2-mercaptoethanol, 2mM glutamine and pen/strep antibiotics. Target cells were ^{51}Cr labelled peptide sensitised and unsensitised (control) P815 cells. The ratio of effector:target was 50, 10 and 2 to 1. The assays were performed in 96 well round bottom plates in duplicate.

The r6K6H polytope associated CDL ISCOMATRIX™ pH4.3 induced CTL responses in 3/3 mice for the SYI, YPH and RPQ epitopes and in 1/3 for the TYQ epitope (Figure 14A). The r6H associated CHL ISCOMATRIX™ pH7 induced CTL responses in 3/3 mice for the SYI, YPH and RPQ epitopes and in 2/3 for the TYQ epitope (Figure 14B). Both formulations induced very low responses to the TYQ epitope.

These results show that CTL responses can be induced using r6K6H polytope associated CDL ISCOMATRIX™ pH4.3 and are comparable to responses with r6H associated CHL ISCOMATRIX™ pH7.

EXAMPLE 18

PREPARATION OF DPPC AND DPL LIPOSOMES WITH A NATURALLY NEGATIVELY CHARGED PROTEIN: E6E7

Liposomes were prepared according to the method of Talsma and Crommelin (1992). Briefly, ^{3}H cholesterol was dissolved in methanol, chloroform then lipid added and liposomes allowed to form by solvent evaporation in a rotaflask with gentle swirling. The lipids used were the standard DPPC and the negatively charged DPL. E6E7 was then added to the liposomes and the mixture sonicated then extruded through a 26G needle. The liposomes were of typical appearance by electron microscopy.

After formulation, preparations were purified on a sucrose gradient (10 to 50% sucrose w/v) and fractions analysed for protein and ISCOMATRIX™ (Figure 15). Protein was detected by sandwich EIA for E7 using monoclonal antibodies. ISCOMATRIX™ was determined by detection ^{3}H cholesterol.

The E6E7 in the DPPC liposomes was found predominanlty in fractions 1-3 but very little was present on the gradient which indicated the protein had precipitated (Figure 15A). The protein that was present was probably not associated with the liposome which was found in fractions 2-4. The E6E7 in the DPL Liposome was found throughout the gradient coinciding with the liposome which was also found throughtout the gradient (Figure 15B). The spread of the formulation throughout the gradient was probably indicative of a range of sizes of liposomes but almost all of the protein seems to be associated with the liposomes.

These results show that negatively charged lipids can be used in liposomes to allow association with a negatively charged protein which would not associate with standard liposomes.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

Table 2 Cytokine Analysis of E6E7 Associated ISCOMATRIX™

Stimulated with	Concentration μg	Cytokine pg/ml	
		αIFN	IL5
GSTE7	5	7400	140
GSTE7	1	1050	85
ConA	0.4	2130	74
RPMI	-	<30	4

REFERENCES

Bradford, M.M. (1976) *Anal. Biochem. 36*:207-212.

Cox, J.C. and Coulter, A.R. *Advances in Adjuvant Technology and Application in Animal Parasite Control Utilising Biotechnology*. Chapter 4. Editor Yong, W.K. CRC Press, 1992.

Cox, J.C. and Coulter, A.R. (1997) *Vaccine 15(3)*:248-256.

Cox, J.C. and Coulter, A.R. (1999) *BioDrugs 12(6)*:439-453.

Edwards, S.J., Margetts, M.B., Hocking, D.M., Moloney, M.B.H., Rothel, L.J. and Webb, E.A. (1998). Design of a candidate recombinant therapeutic vaccine or cervical cancer. In: Recent Research Developmens in Biotechnology & Bioengineering. Editor S.G. Pandalai, Research Signpost, India, 343-356.

Elliot, S.L., Pye, S., Le, T., Mateo, L., Cox, J., Macdonald, L., Scalzo, A.A., Forbes, C.A. and Suhrbier, A. (1999) *Vaccine 17*:2009-2019.

Morein, B., Lövgren, K. and Hoglund, S., (1989), Immunostimulating complex (ISCOM). In "Vaccines: Recent Trends and Progress." G. Gregoriadis, A.C. Allison and G. Poster (Eds), Plenium Press, New York, p153.

Talsma, H. and Crommelin, D.J.A. (1992) *BioPharm*, October, 36-47.

Valerio, R.M., Bray, A.M. and Maeji, N.J., *Int. J. Pept. Prof. Res. 44*:158-165 (1994).

CLAIMS:

1. An immunogenic complex comprising a charged organic carrier and a charged antigen, which organic carrier and antigen are electrostatically associated.

2. The immunogenic complex according to claim 1 wherein said carrier is negatively charged and said antigen is positively charged.

3. The immunogenic complex according to claim 2 wherein said antigen is a protein or derivative or equivalent thereof.

4. The immunogenic complex according to claim 2 wherein said carrier is an adjuvant or derivative or equivalent thereof.

5. The immunogenic complex according to claim 2 wherein said antigen is a protein or derivative or equivalent thereof and said carrier is an adjuvant or derivative or equivalent thereof.

6. The immunogenic complex according to claim 5 wherein said negatively charged adjuvant is a naturally negatively charged adjuvant which has been modified to increase the degree of its negative charge.

7. The immunogenic complex according to claim 5 wherein said positively charged protein is a naturally positively charged protein which has been modified to increase the degree of its positive charge.

8. The immunogenic complex according to claim 5 wherein said negatively charged adjuvant is a naturally negatively charged adjuvant which has been modified to increase the degree of its negative charge and said positively charged protein is a naturally positively charged protein which has been modified to increase the degree of its positive charge.

9. The immunogenic complex according to any one of claims 5-8 wherein said adjuvant comprises a saponin.

8. The immunogenic complex according to any one of claims 5-8 wherein said adjuvant is a saponin complex.

11. The immunogenic complex according to claim 10 wherein said saponin complex is ISCOMATRIX™.

12. The immunogenic complex according to any one of claims 5-8 wherein said adjuvant comprises a phospholipid.

13. The immunogenic complex according to claim 12 wherein said phospholipid is a phosphoglyceride.

14. The immunogenic complex according to claim 13 wherein the phosphoglyceride is selected from the group consisting of phosphatidyl inositol, phosphatidyl glycerol, phosphatidic acid and cardiolipin.

15. The immunogenic complex according to claim 12 wherein said phospholipid is lipid A.

16. The immunogenic complex according to claim 15 wherein the lipid A is selected from the group consisting of diphosphoryl lipid A such as OM174 and monophosphoryl lipid A.

17. The immunogenic complex according to any one of claims 1-16 wherein said complex induces a cytotoxic T-lymphocyte response.

18. A vaccine composition comprising as the active component a charged organic carrier and a charged antigen, which carrier and antigen are electrostatically associated, together with one or more pharmaceutically acceptable carriers and/or diluents.

19. The vaccine composition according to claim 18 wherein said carrier is negatively charged and said antigen is positively charged.

20. The vaccine composition according to claim 19 wherein said antigen is a protein or derivative or equivalent thereof.

21. The vaccine composition according to claim 19 wherein said carrier is an adjuvant or derivative or equivalent thereof.

22. The vaccine composition according to claim 19 wherein said antigen is a protein or derivative or equivalent thereof and said carrier is an adjuvant or derivative or equivalent thereof.

23. The vaccine composition according to claim 22 wherein said negatively charged adjuvant is a naturally negatively charged adjuvant which has been modified to increase the degree of its negative charge.

24. The vaccine composition according to claim 22 wherein said positively charged protein is a naturally positively charged protein which has been modified to increase the degree of its positive charge.

25. The vaccine composition according to claim 22 wherein said negatively charged adjuvant is a naturally negatively charged adjuvant which has been modified to increase the degree of its negative charge and said positively charged protein is a naturally positively charged protein which has been modified to increase the degree of its positive charge.

26. The vaccine composition according to any one of claims 22-25 wherein said adjuvant comprises a saponin.

27. The immunogenic complex according to any one of claims 22-25 wherein said adjuvant is a saponin complex.

28. The vaccine composition according to claim 27 wherein said saponin complex is ISCOMATRIX™.

29. The vaccine composition according to any one of claims 22-25 wherein said adjuvant comprises a phospholipid.

30. The vaccine composition according to claim 29 wherein said phospholipid is a phosphoglyceride.

31. The vaccine composition according to claim 30 wherein the phosphoglyceride is selected from the group consisting of phosphatidyl inositol, phosphatidyl glycerol, phosphatidic acid and cardiolipin.

32. The vaccine composition according to claim 29 wherein said phospholipid is lipid A.

33. The vaccine composition according to claim 32 wherein the lipid A is selected from the group consisting of diphosphoryl lipid A such as OM174 and monophosphoryl lipid A.

34. The vaccine composition according to any one of claims 18-33 wherein said composition induces a cytotoxic T-lymphocyte response.

35. A method of eliciting, inducing or otherwise facilitating, in a mammal, an immune response to an antigen said method comprising administering to said mammal an effective amount of an immunogenic complex according to any one of claims 1-17.

36. A method of eliciting, inducing or otherwise facilitating, in a mammal, an immune response to an antigen said method comprising administering to said mammal an effective amount of a vaccine composition according to any one of claims 18-34.

37. The method according to claim 17 or 36 wherein said immune response comprises a cytotoxic T-lymphocyte response.

38. A method of treating a disease condition in a mammal said method comprising administering to said mammal an effective amount of an immunogenic complex according to any one of claims 1-17 wherein administering said complex elicits, induces or otherwise facilitates an immune response which inhibits, halts, delays or prevents the onset or progression of said disease condition.

39. A method of treating a disease condition in a mammal said method comprising administering to said mammal an effective amount of a vaccine composition according to any one of claims 18-34 wherein administering said composition elicits, induces or otherwise facilitates an immune response which inhibits, halts, delays or prevents the onset or progression of the disease condition.

40. The method according to claim 38 or 39 wherein said immune response comprises a cytotoxic T-lymphocyte response.

41. The method according to any one of claims 38-41 wherein said treatment is therapeutic or prophylactic.

42. The method according to any one of claims 38-41 wherein said disease condition results from a microbial infection or a cancer.

43. The method according to claim 43 wherein said microbial infection is HIV, Hepatitis B, Hepatitis C, tuberculosis or a parasitic condition and said cancer is melanoma, prostate cancer or breast cancer.

44. Use of an immunogenic complex according to any one of claims 1-17 in the manufacture of a medicament for inhibiting, halting, delaying or preventing the onset or progression of a disease condition.

45. Use of a vaccine composition according to any one of claims 18-34 in the manufacture of a medicament for inhibiting, halting, delaying or preventing the onset or progression of a disease condition.

46. Use according to claim 44 or 45 wherein said disease condition results from a microbial infection or a cancer.

47. Use according to claim 46 wherein said microbial infection is HIV, Hepatitis B, Hepatitis C, tuberculosis or a parasitic infection and said cancer is melanoma, prostate cancer or breast cancer.

48. An agent for use in inhibiting, halting, delaying or preventing the onset or progression of a disease condition wherein said agent comprises an immunogenic complex according to any one of claims 1-17.

49. An agent for use in inhibiting, halting, delaying or preventing the onset or progression of a disease condition wherein said agent comprises a vaccine composition according to any one of claims 18-34.

50. An agent according to claim 48 or 49 wherein said disease condition results from a microbial infection or a cancer.

51. An agent according to claim 50 wherein said microbial infection is HIV, Hepatitis B, Hepatitis C, tuberculosis or a parasitic infection and said cancer is melanoma, prostate cancer or breast cancer.

ABSTRACT

The present invention relates generally to an immunogenic complex comprising a charged organic carrier and a charged antigen and, more particularly, a negatively charged organic carrier and a positively charged antigen. The complexes of the present invention are useful, *inter alia*, as therapeutic and/or prophylactic agents for facilitating the induction of a cytotoxic T-lymphocyte response to an antigen.

Figure 1

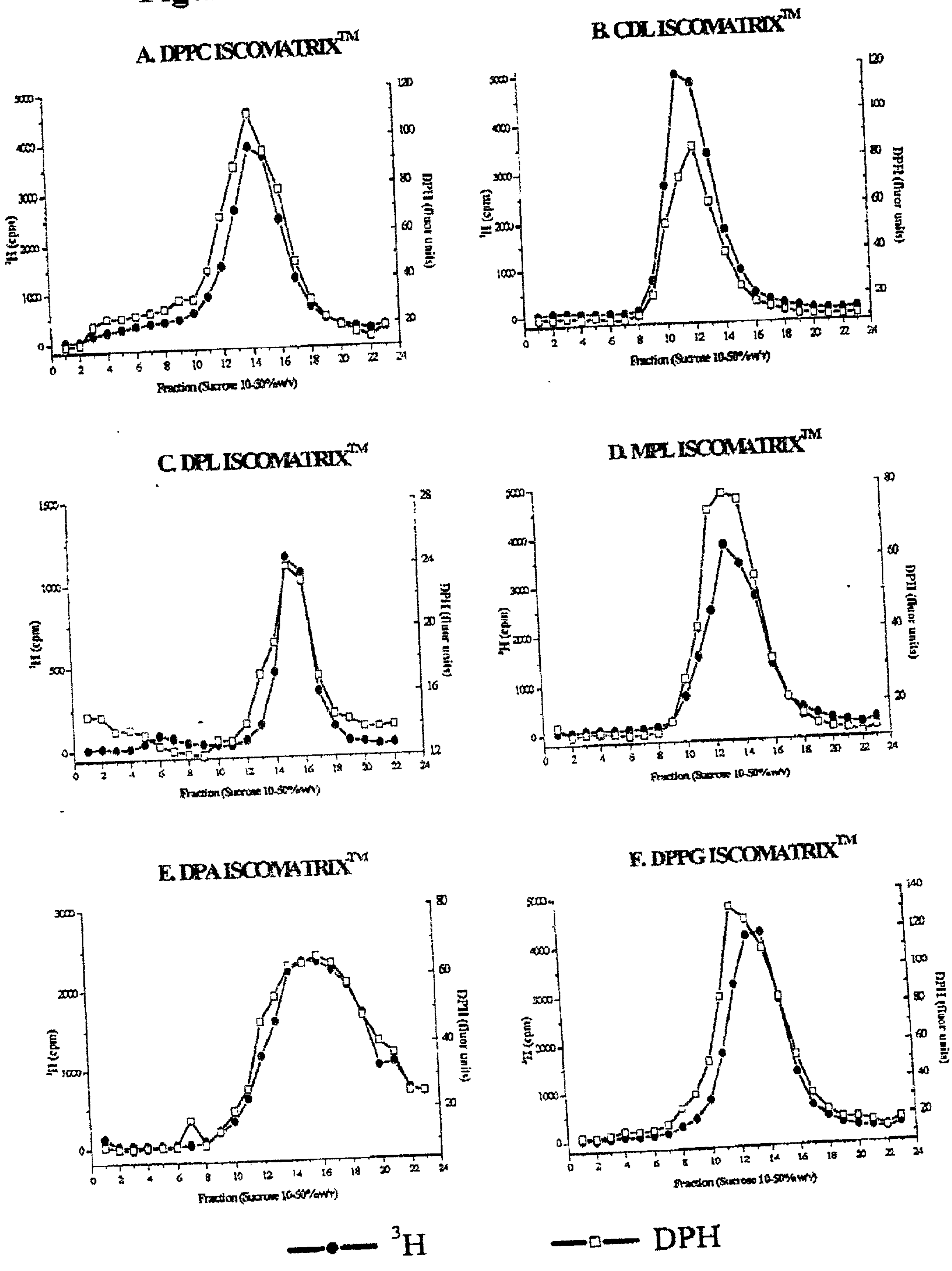
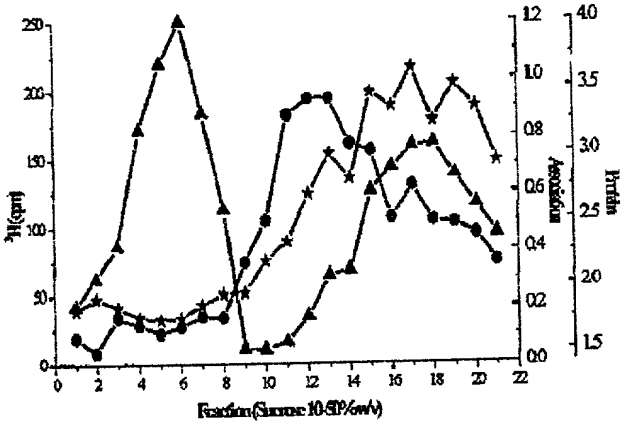
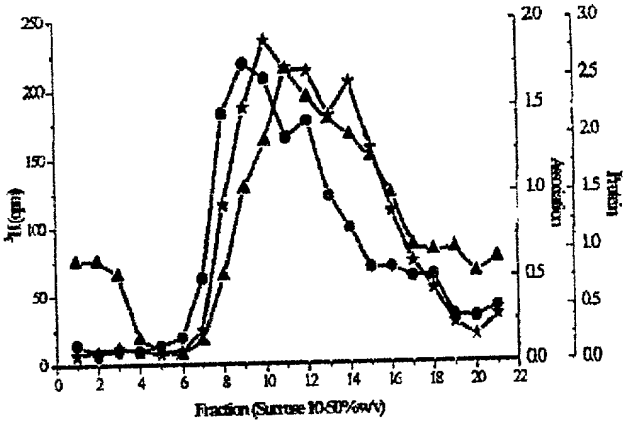


Figure 2

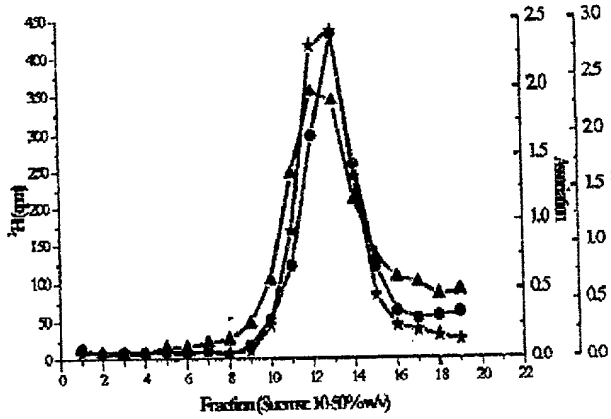
A. HpE+DPPCISCOMATRIX™



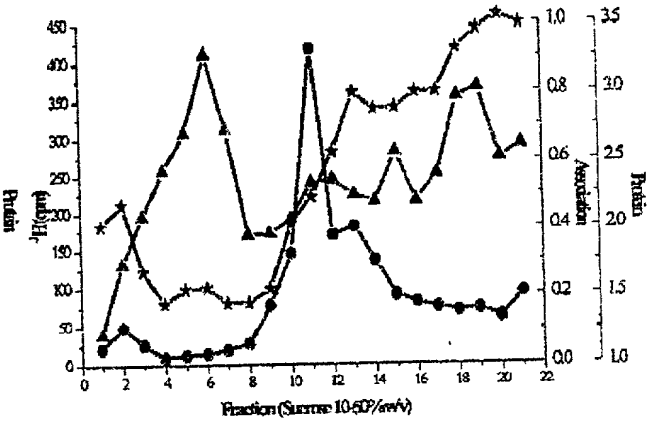
B. HpE+CDLISCOMATRIX™



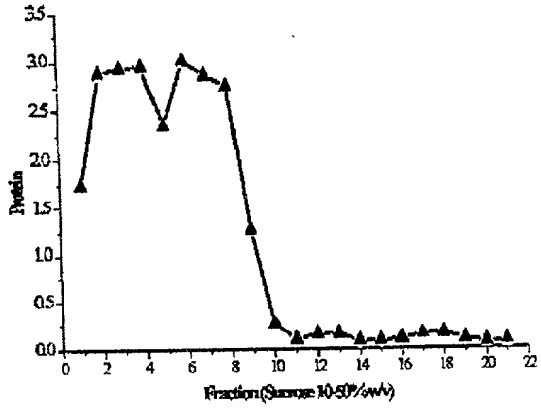
C. HpE+DPLISCOMATRIX™



D. HpE+DPPGISCOMATRIX™



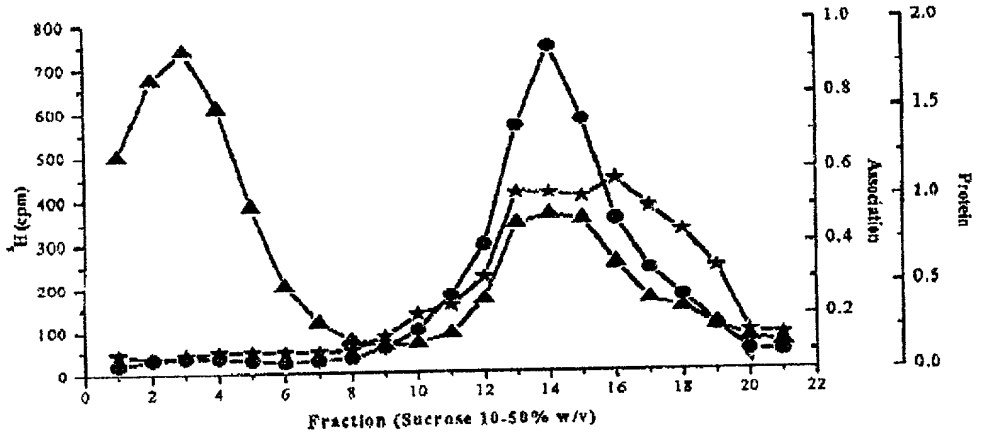
E. HpE



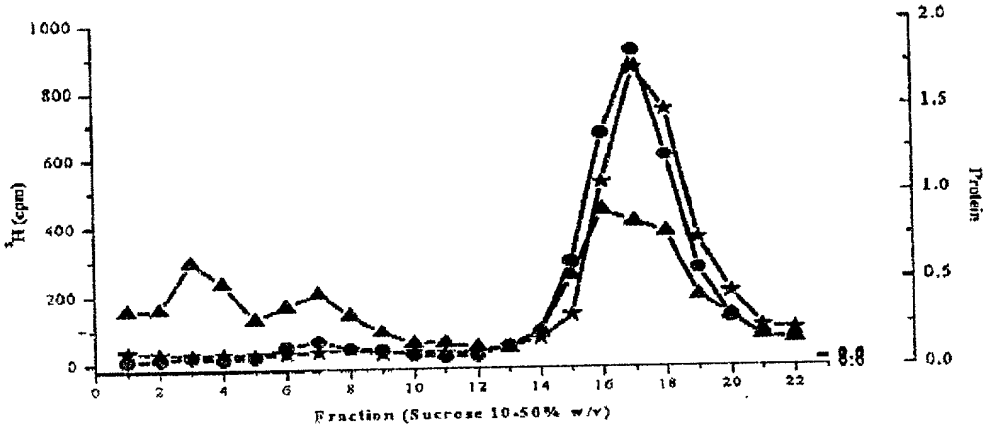
—●— ^{3}H —▲— Protein —★— Association

Figure 3

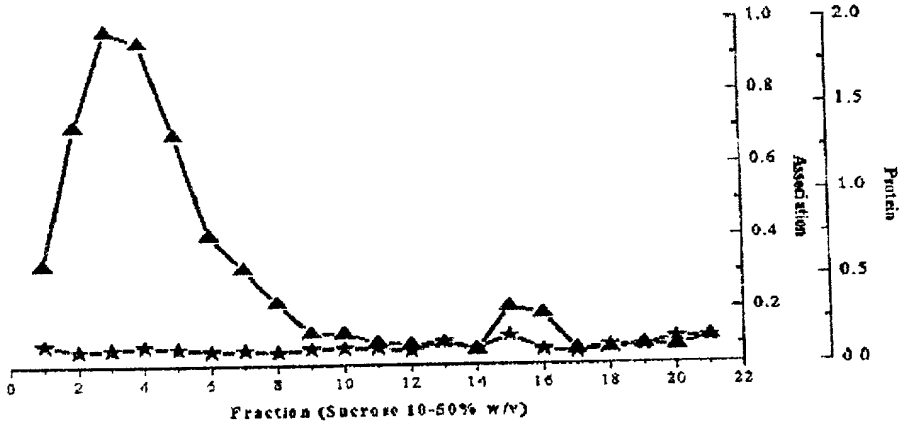
A. ESO + DPPC ISCOMATRIX™



B. ESO + DPL ISCOMATRIX™



C. ESO



—●— ^{3}H —▲— Protein —★— Association

Figure 4

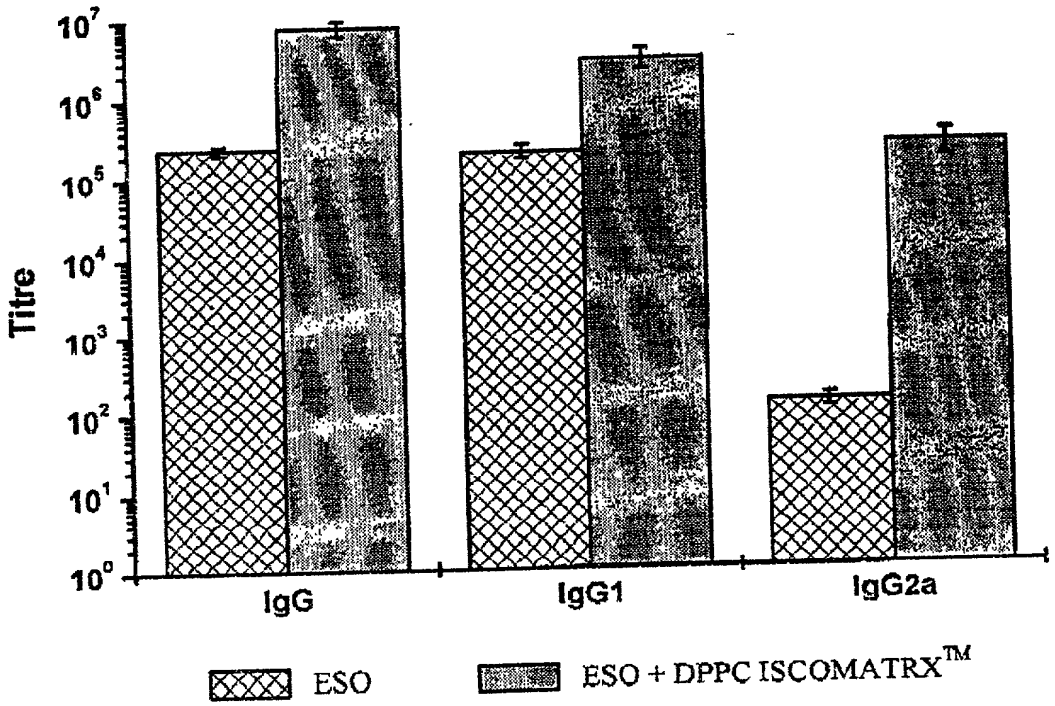


Figure 5

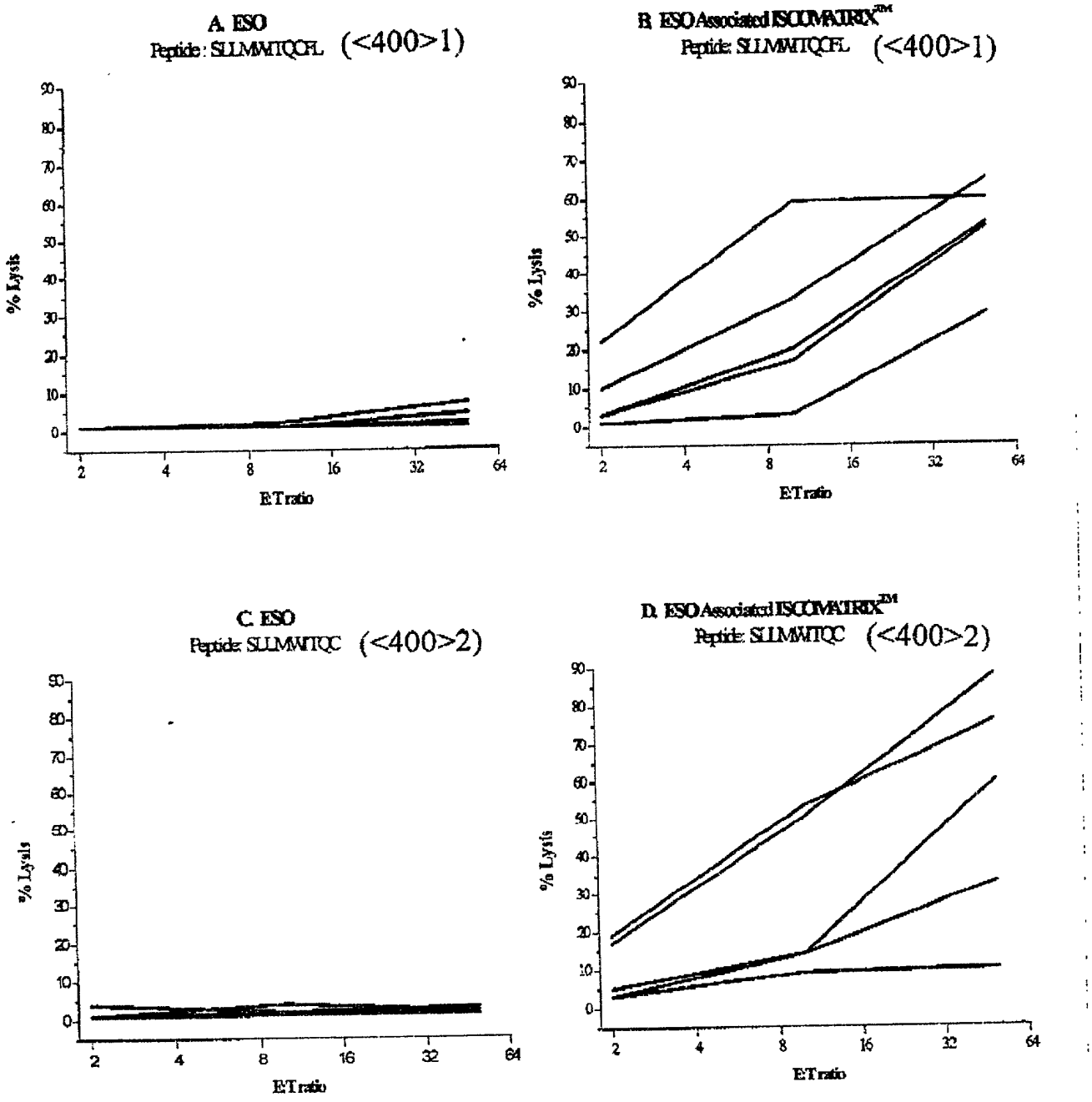
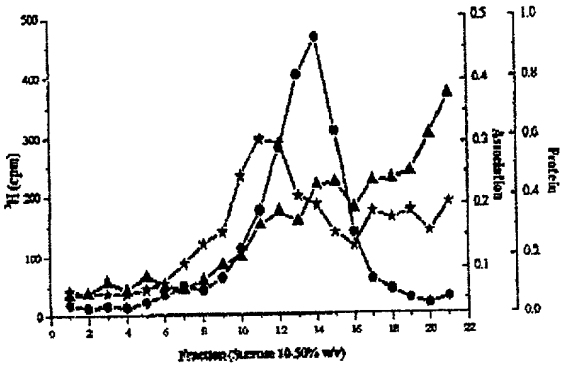
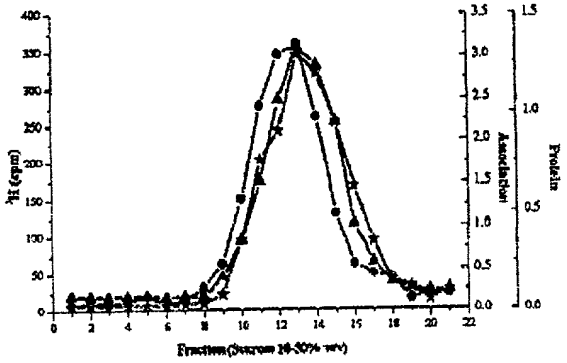


Figure 6

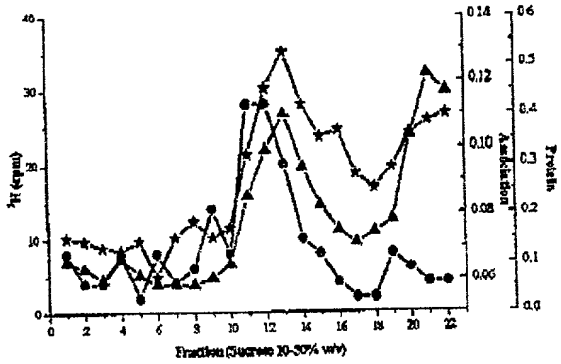
A. E6E7 + DPPC ISCOMATRIX™



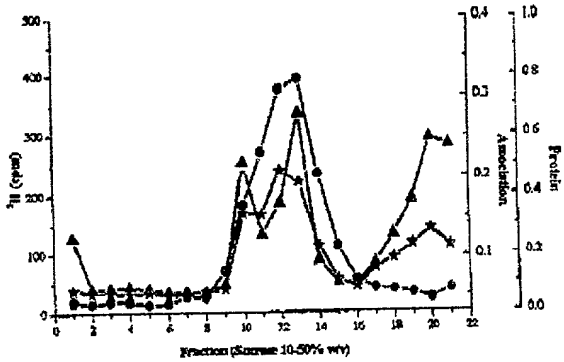
B. E6E7 + CDL ISCOMATRIX™



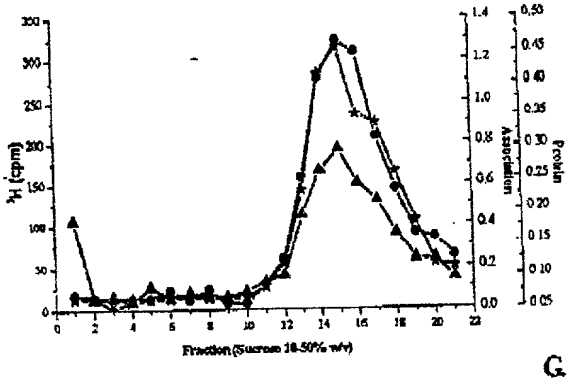
C. E6E7 + DPL ISCOMATRIX™



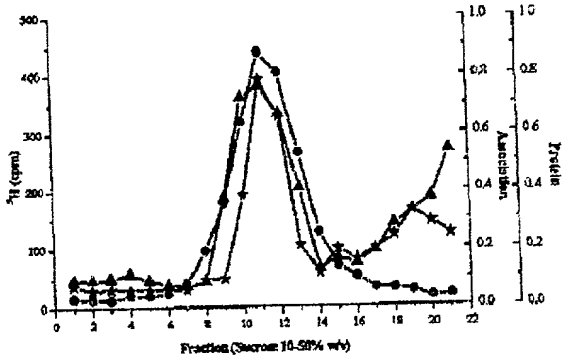
D. E6E7 + MPL ISCOMATRIX™



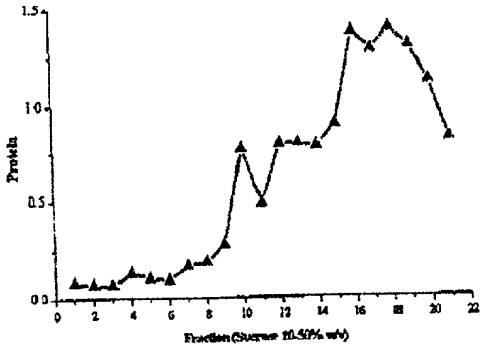
E. E6E7 + DPA ISCOMATRIX™



F. E6E7 + DPPG ISCOMATRIX™



G. E6E7



—●— ^{3}H —▲— Protein —★— Association

Figure 7

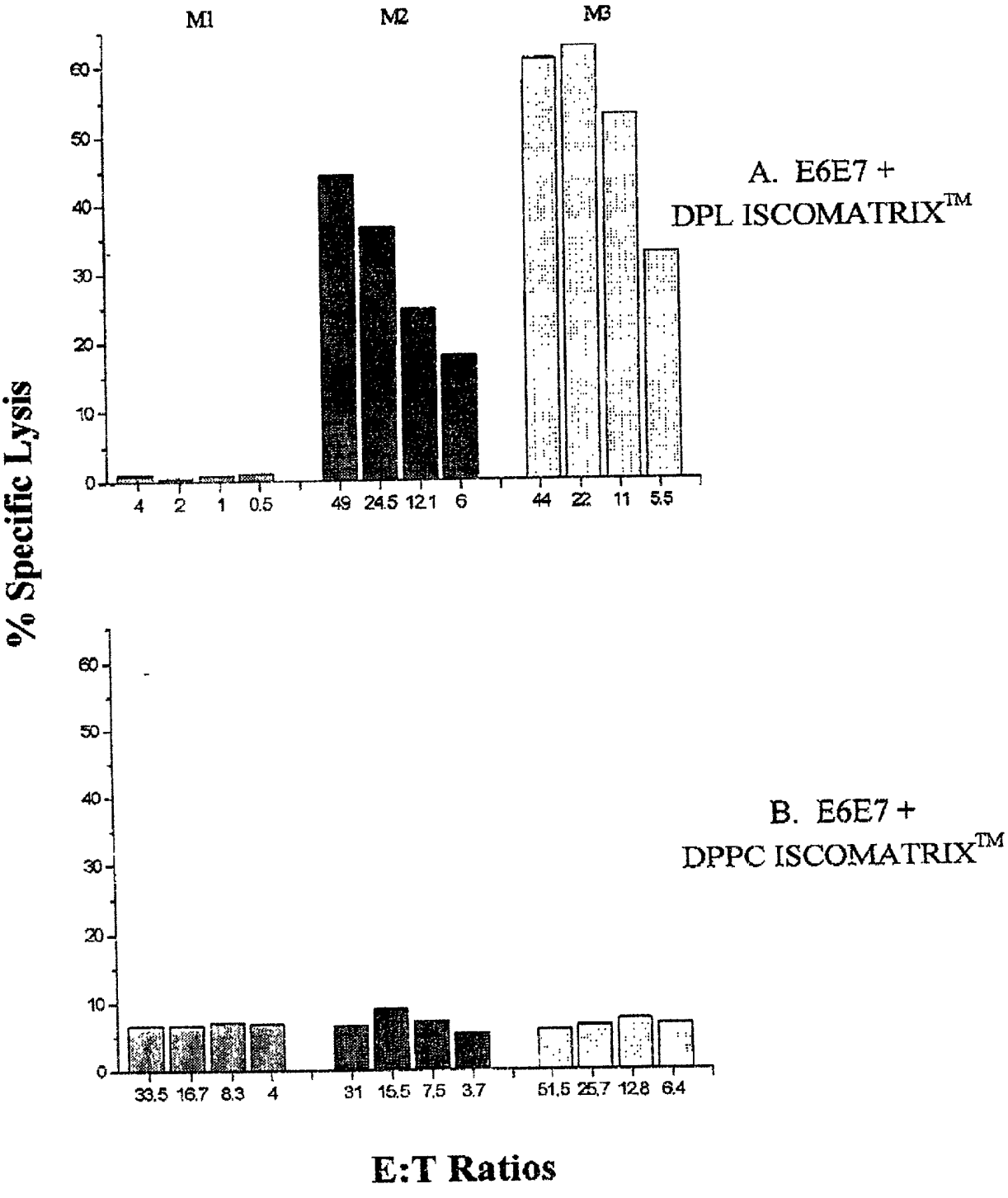
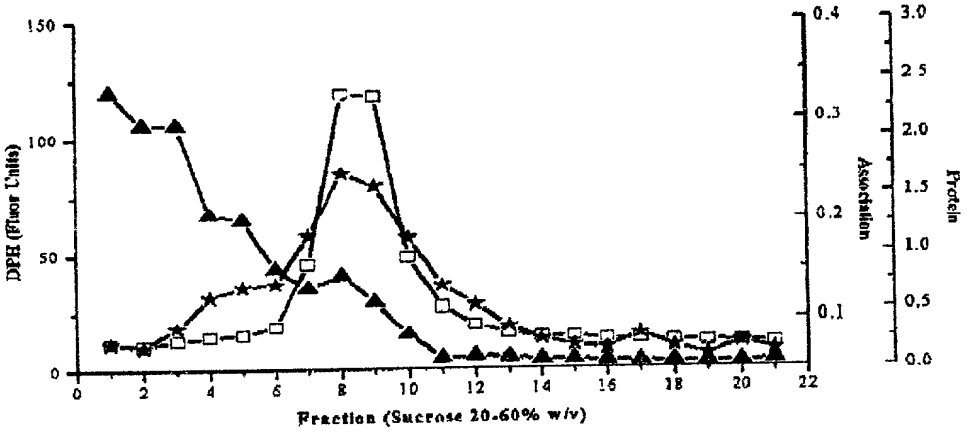
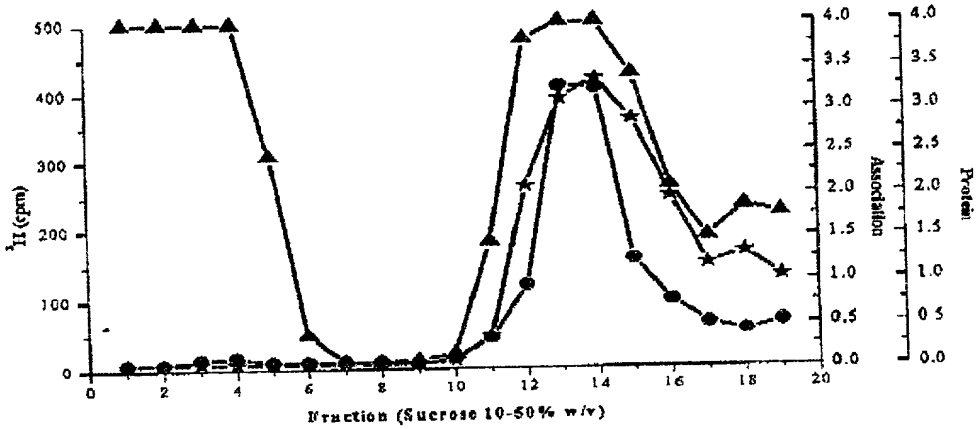


Figure 8

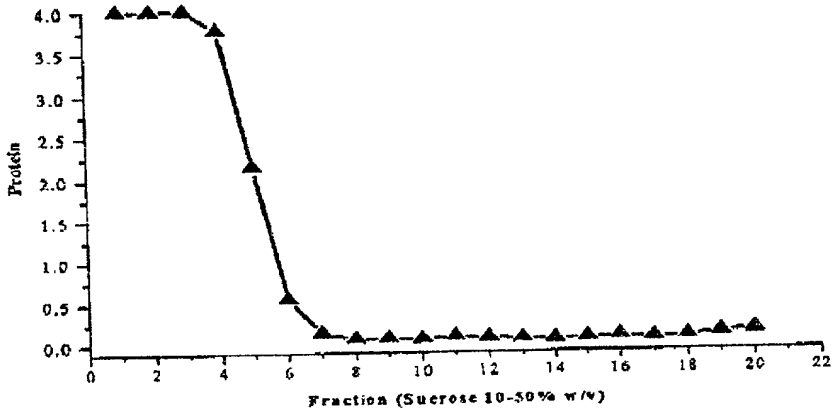
A. HpC + DPPC ISCOMATRIX™



B. HpC + DPL ISCOMATRIX™



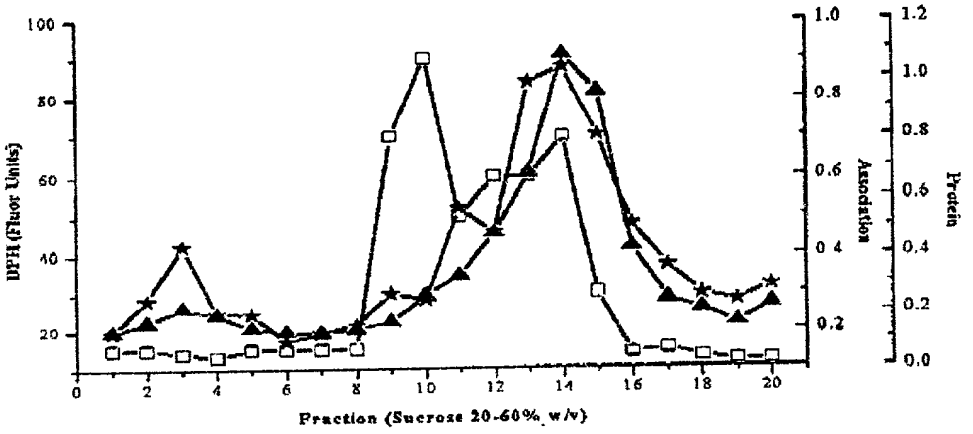
C. HpC



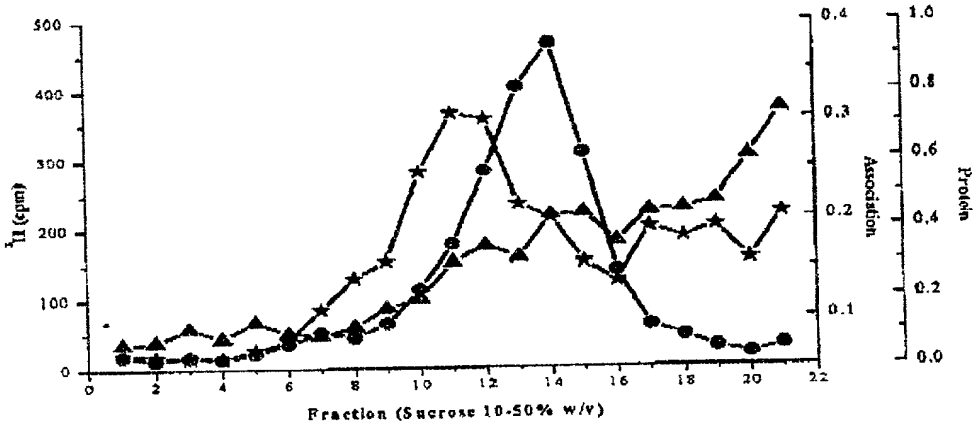
DPH ^{3}H Protein Association

Figure 9

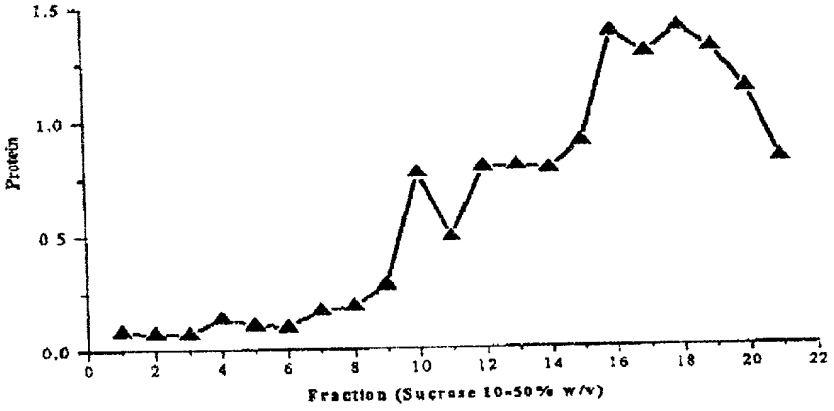
A. E6E7 + DPPC ISCOMATRIX™ pH 6



B. E6E7 + DPPC ISCOMATRIX™ pH 7.2



C. E6E7

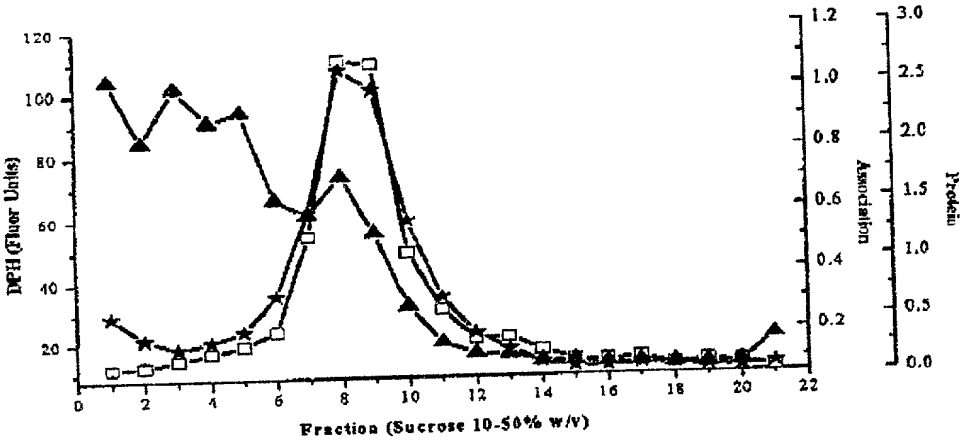


DPH ³H

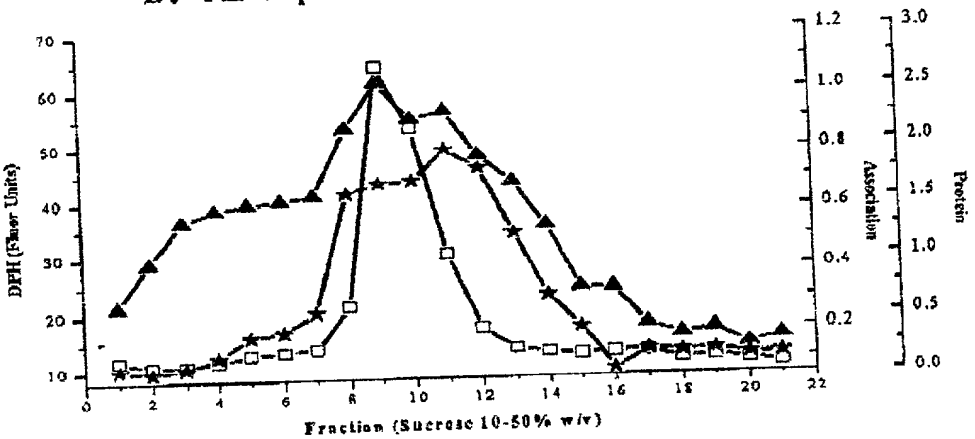
Protein Association

Figure 10

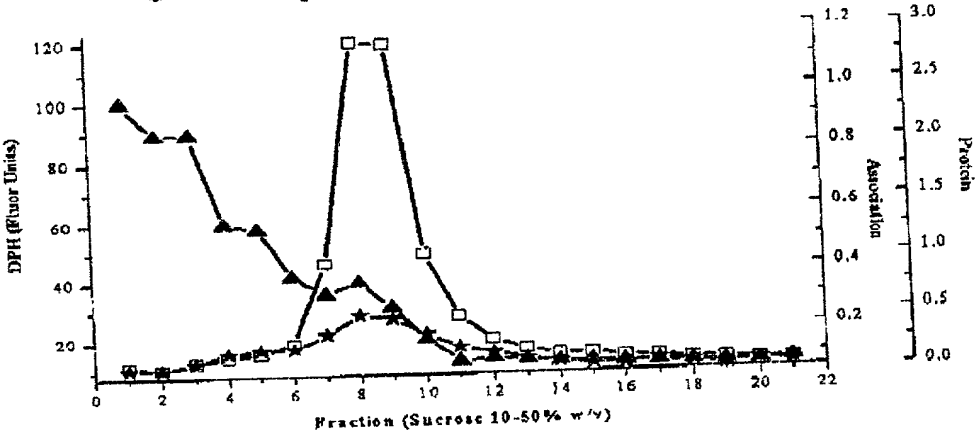
A. 6K6H-HpC + DPPC ISCOMATRIX™



B. 6H-HpC + CHL ISCOMATRIX™



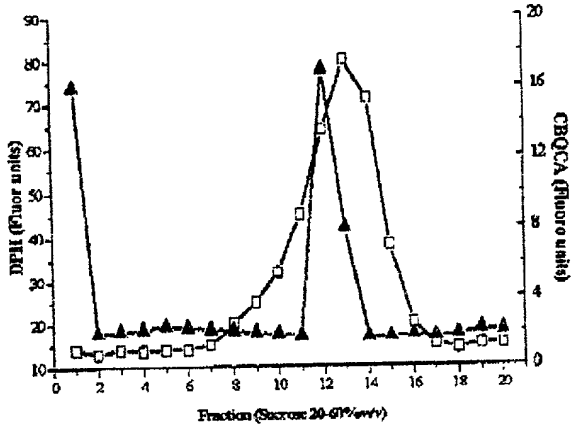
C. 6H-HpC + DPPC ISCOMATRIX™



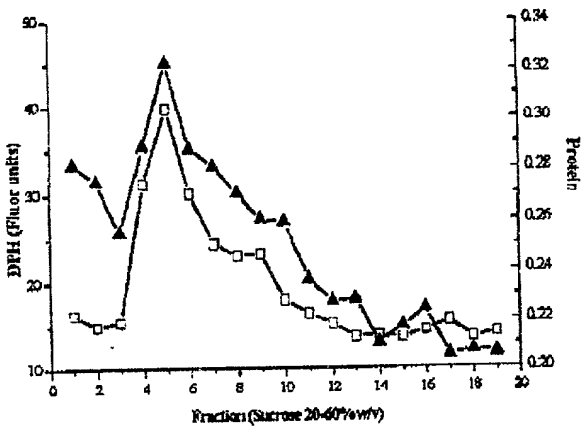
—□— DPH —▲— Protein —★— Association

Figure 11

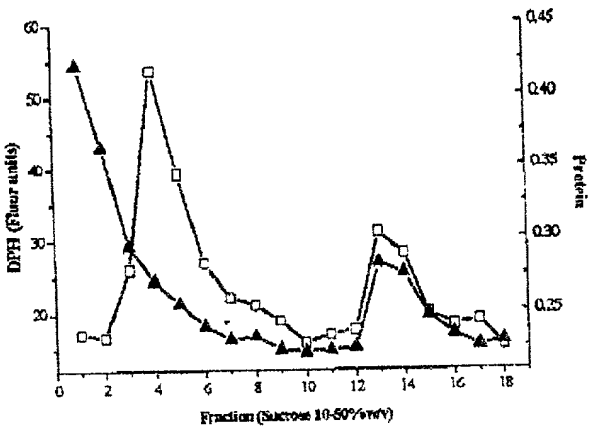
A. PAL polytope ISCOM™



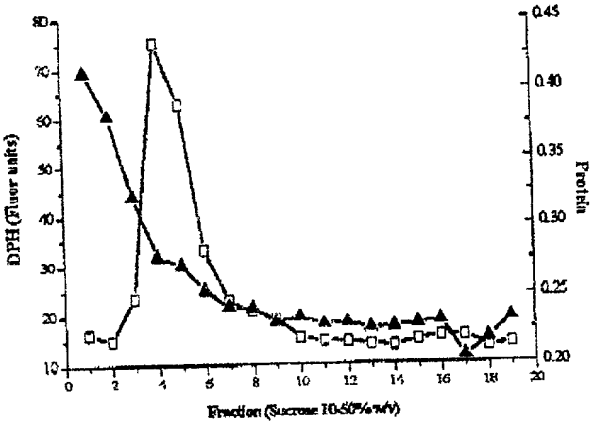
B. 6H polytope + Chelating ISCOMATRIX™



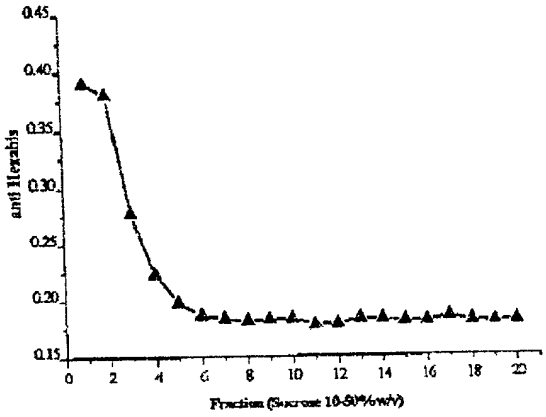
C. 6K polytope + DPPC ISCOMATRIX™



D. No tag polytope + DPPC ISCOMATRIX™



E. No tag polytope



—□— DPH —▲— Protein

Figure 12A

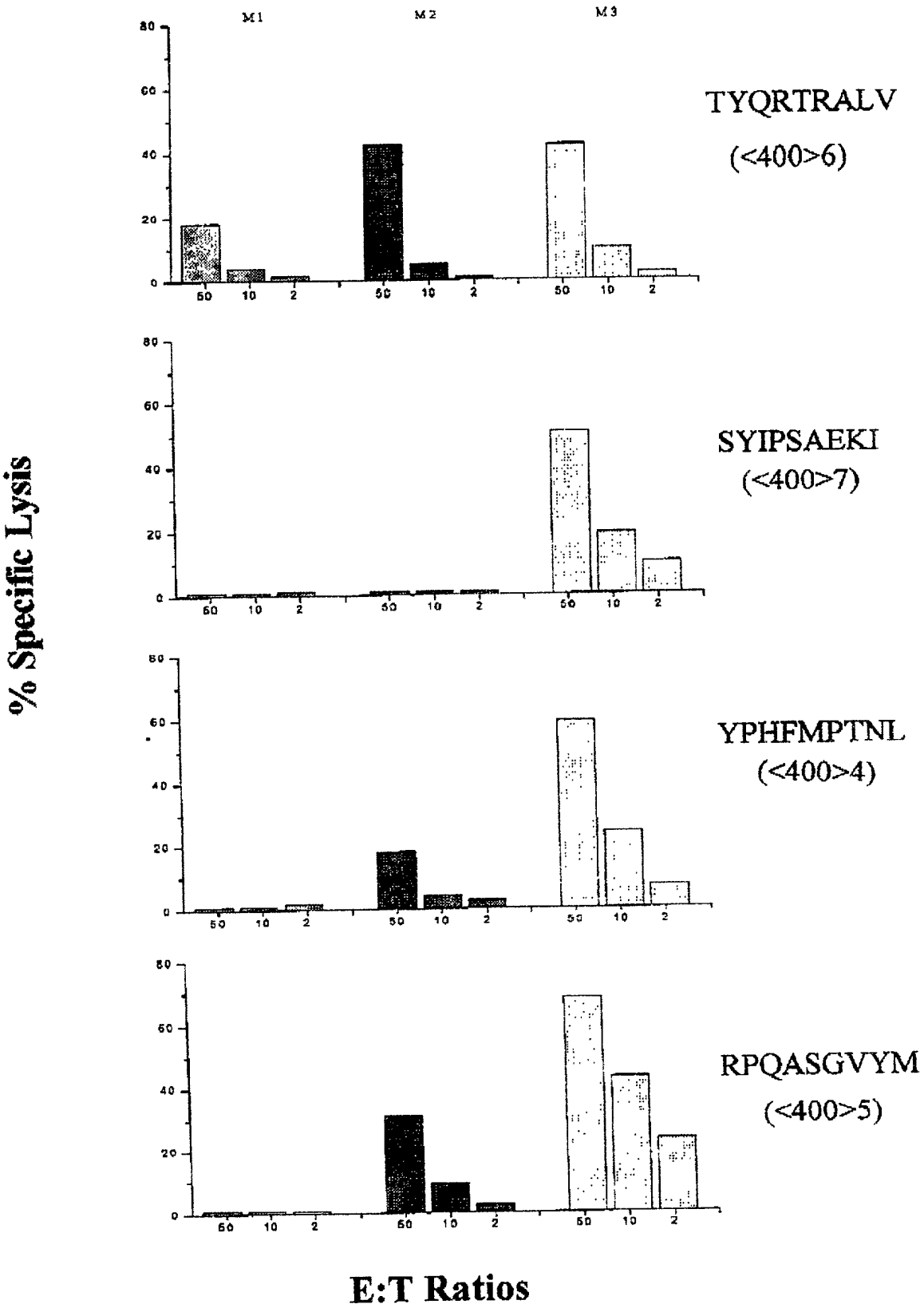


Figure 12B

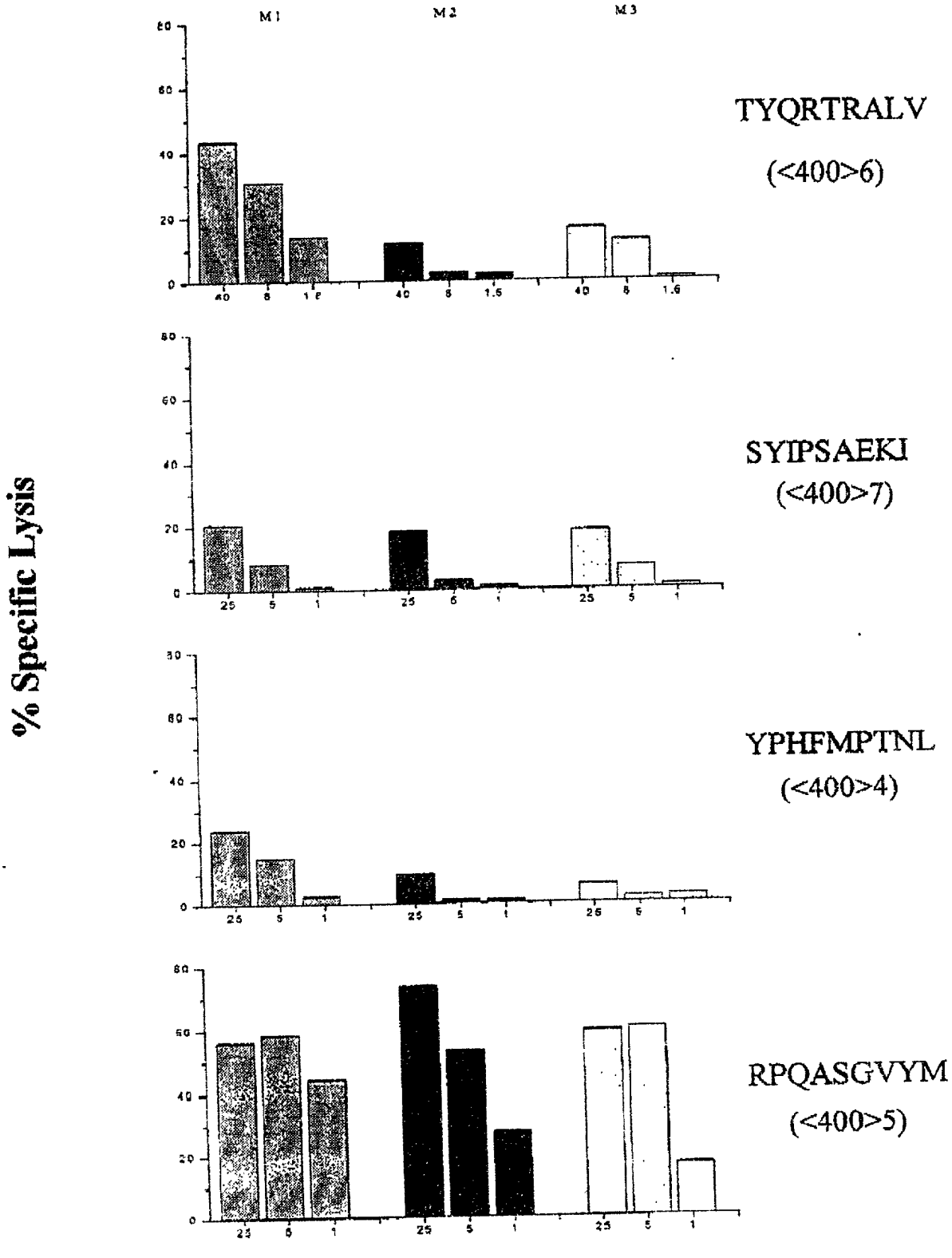


Figure 12C

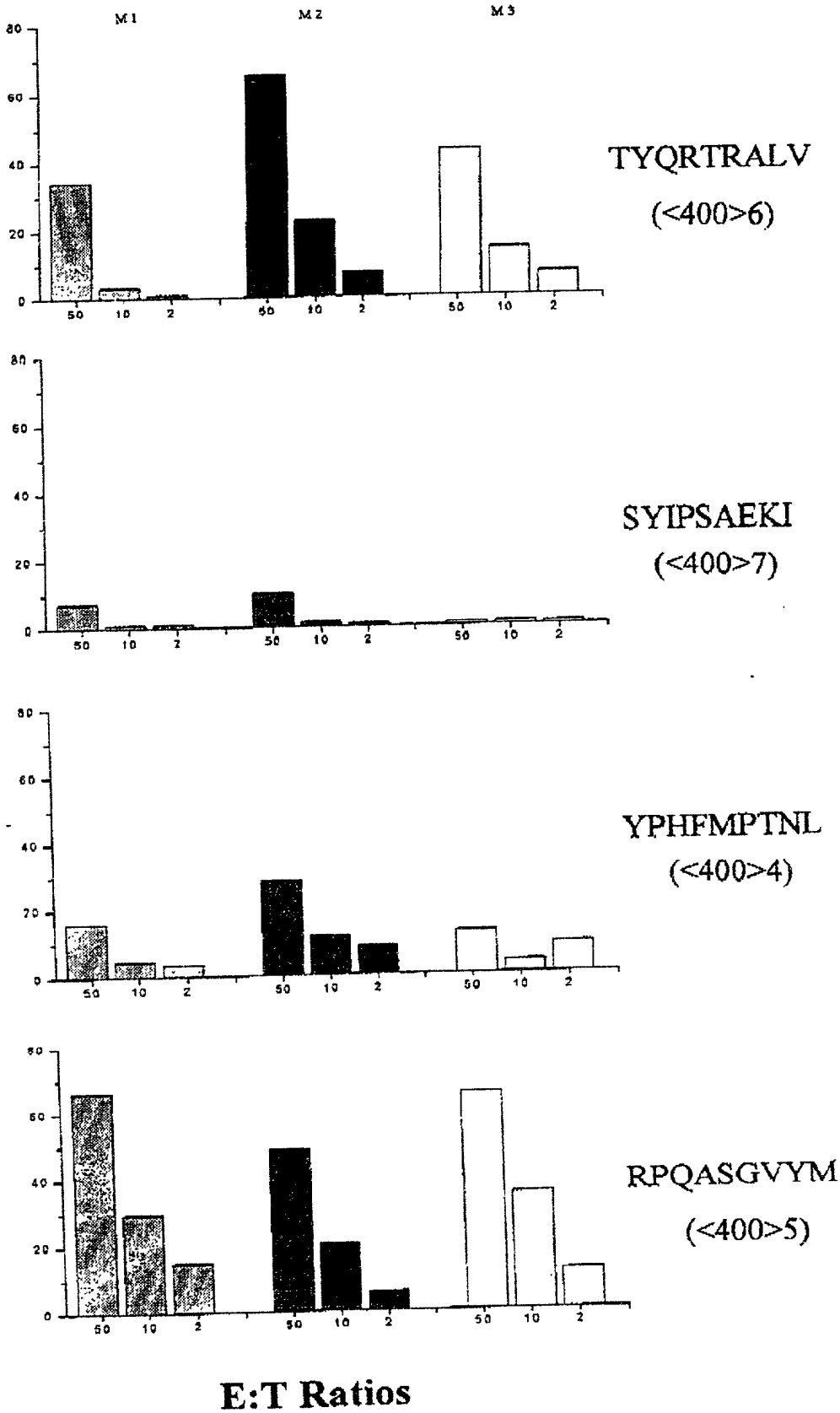


Figure 12D

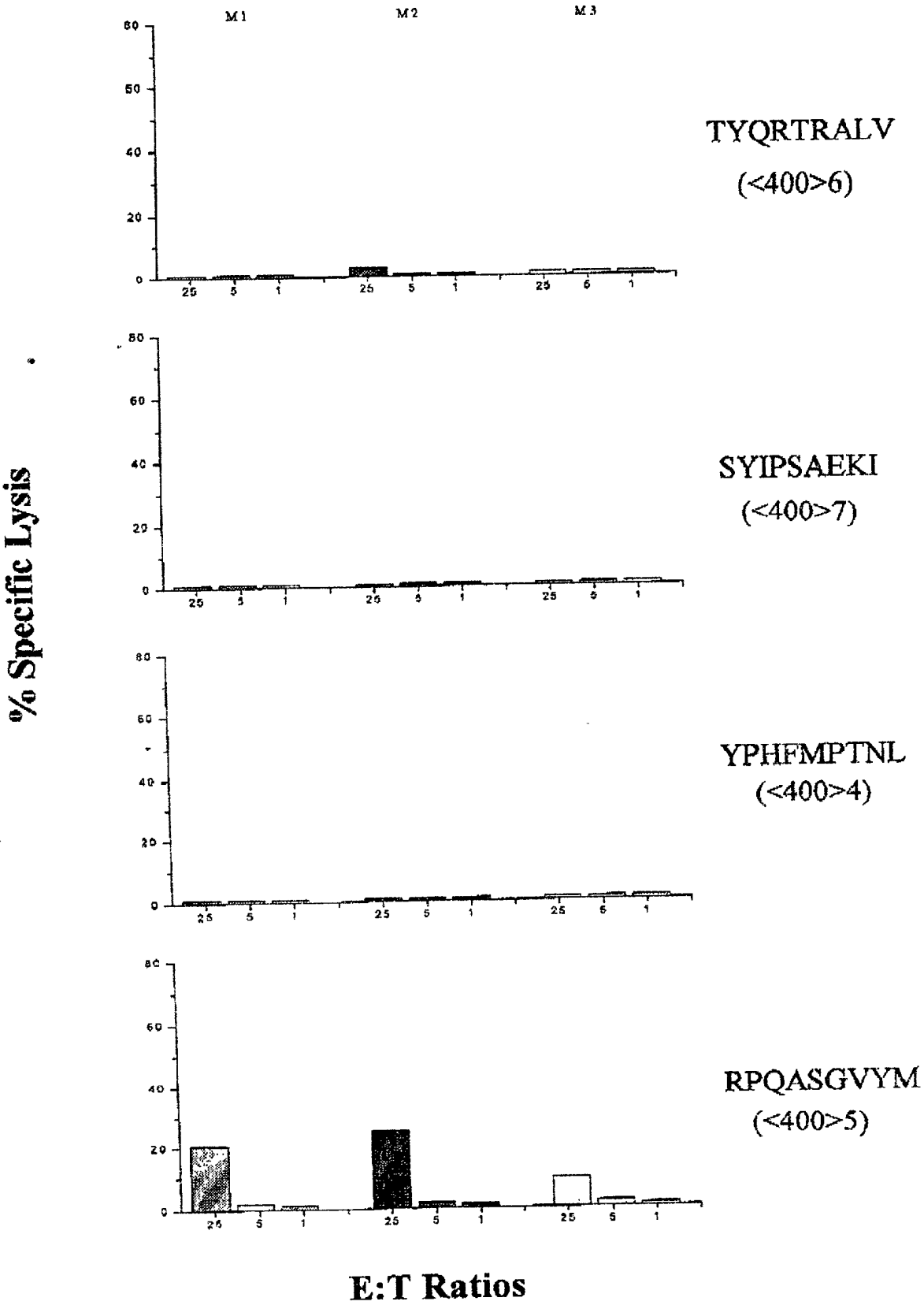
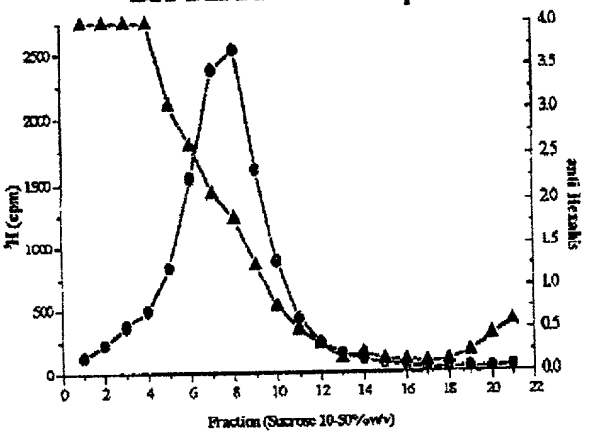
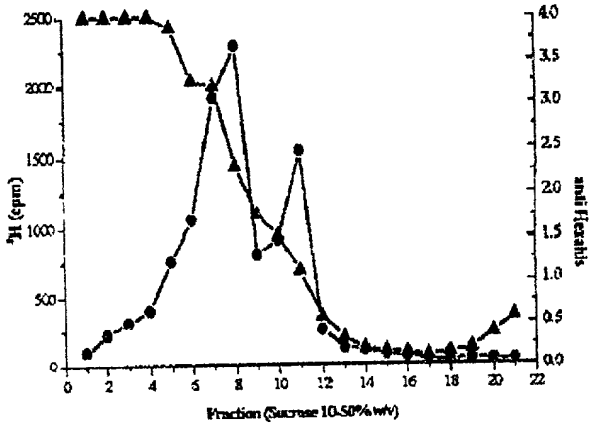


Figure 13

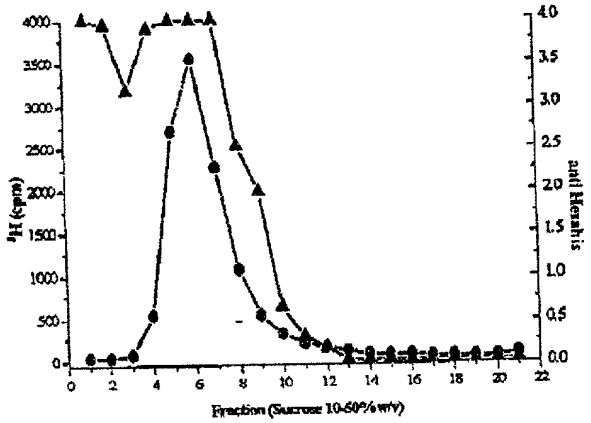
A. r6K6H polytope + DPPC ISCOMATRIX™ pH 7



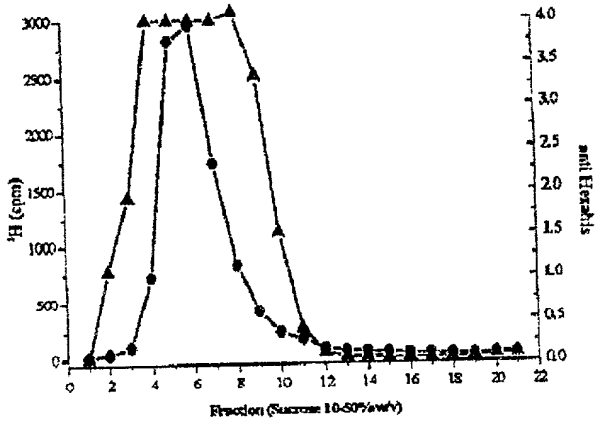
B. r6K6H polytope + DPPC ISCOMATRIX™ pH 4.3



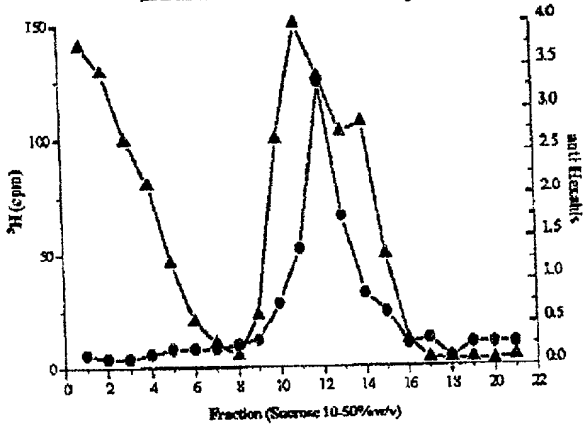
C. r6K6H polytope + CDL ISCOMATRIX™ pH 7



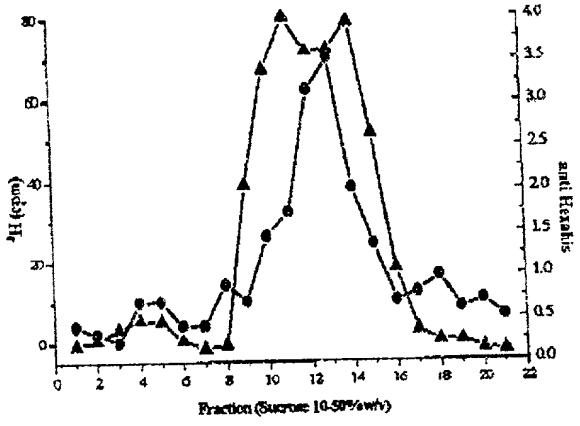
D. r6K6H polytope + CDL ISCOMATRIX™ pH 4.3



E. r6K6H polytope + DPL ISCOMATRIX™ pH 7



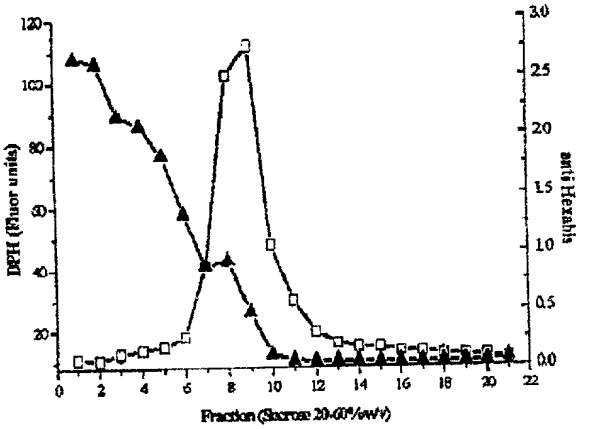
F. r6K6H polytope + DPL ISCOMATRIX™ pH 4.3



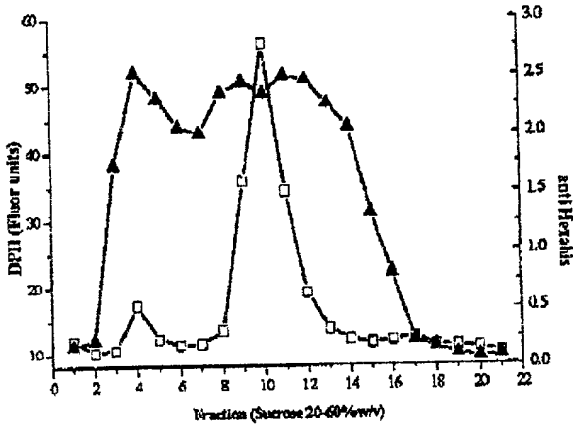
—●— ^{3}H —▲— Hexahis

Figure 13

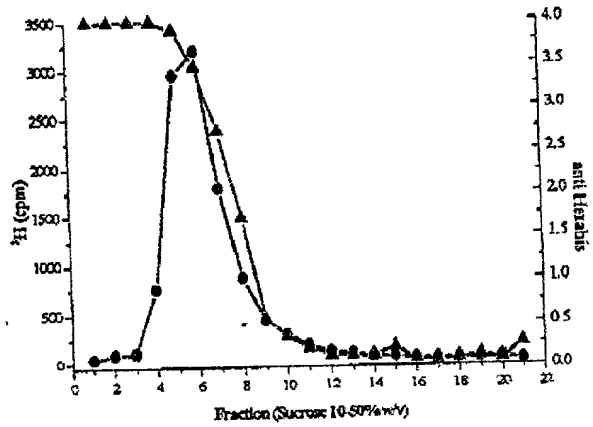
G. r6H polytope + DPPC ISCOMATRIX™



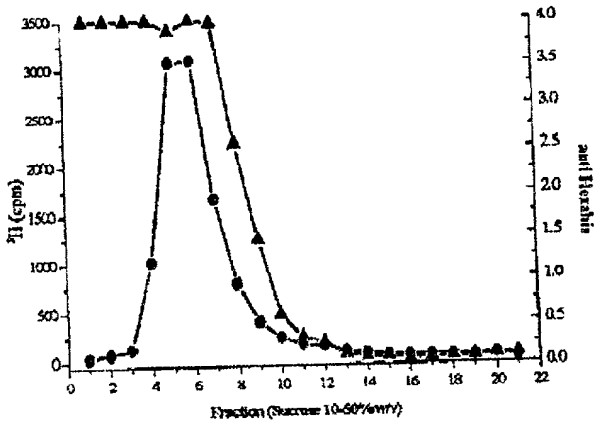
H. r6H polytope + Chelating ISCOMATRIX™



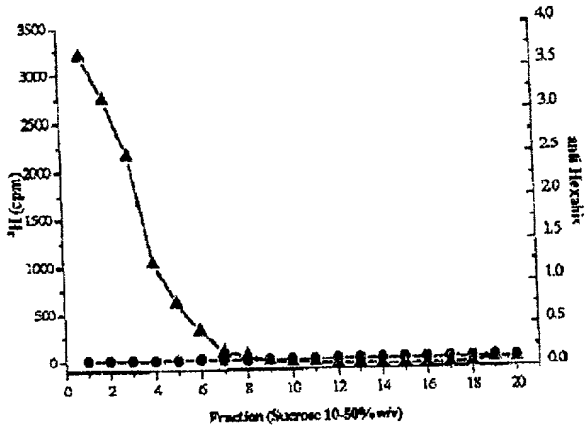
I. r6H polytope + CDL ISCOMATRIX™ pH 7



J. r6H polytope + CDL ISCOMATRIX™ pH 4.3



K. r6H6K polytope



—●— ^{3}H —□— DPH —▲— Hexahis

Figure 14A

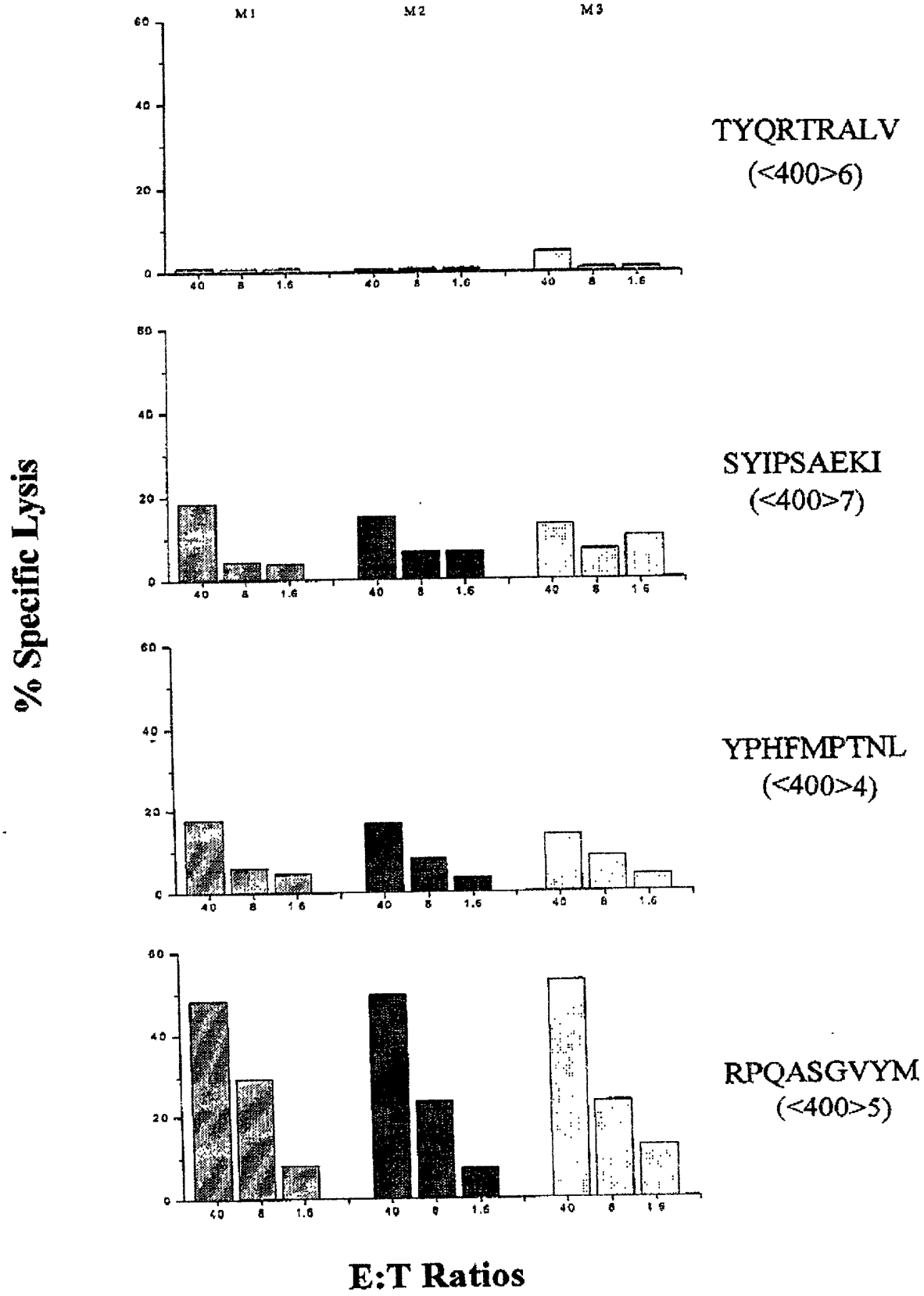


Figure 14B

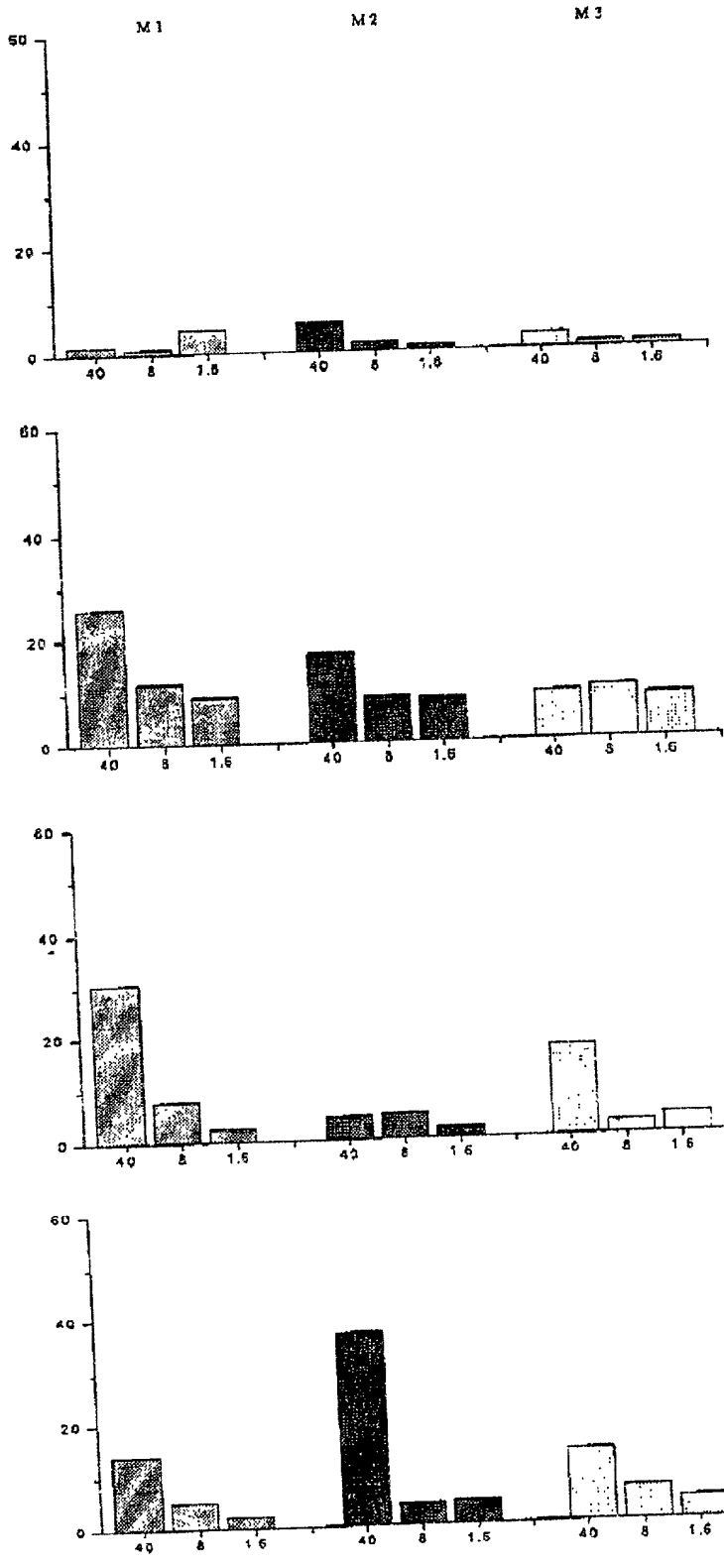
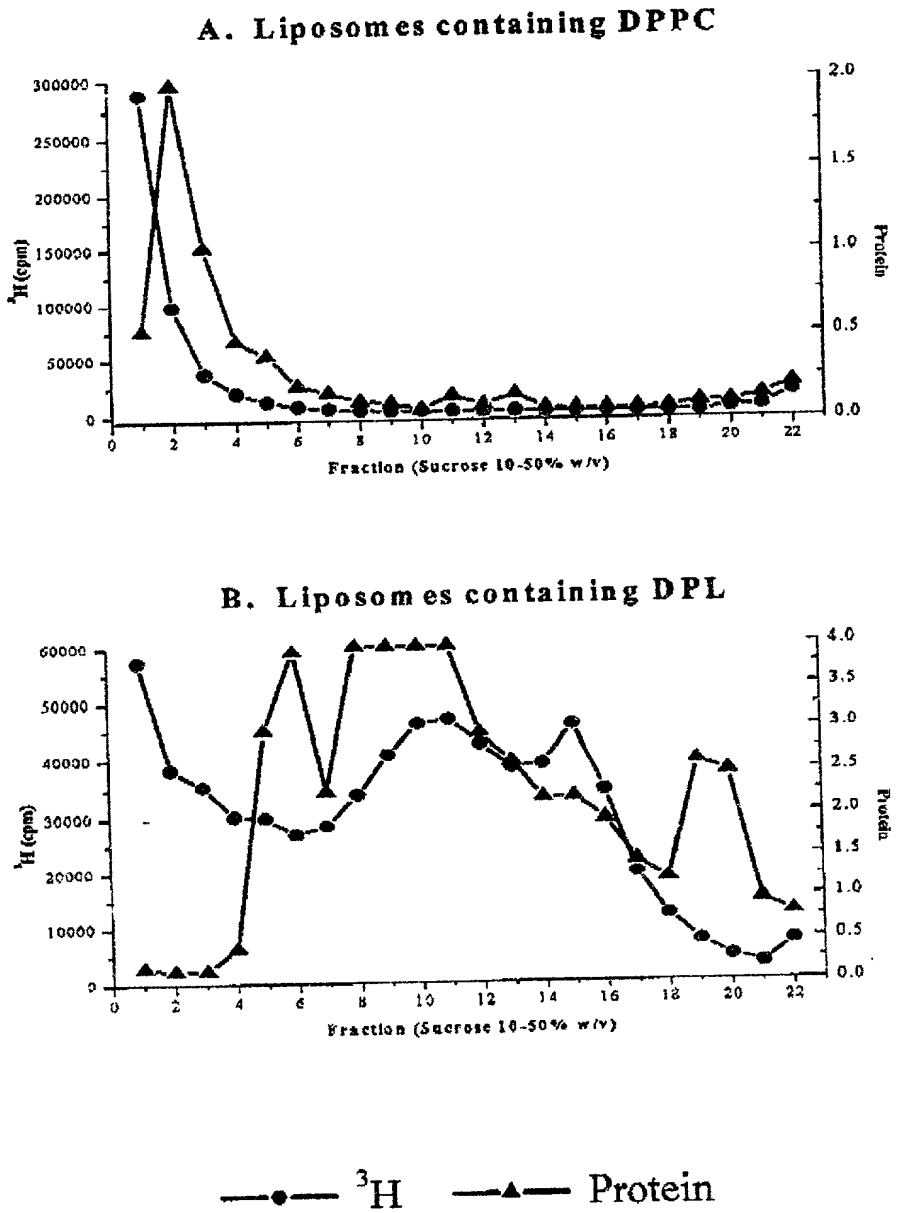


Figure 15



DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I HEREBY DECLARE:

THAT my residence, post office address, and citizenship are as stated below next to my name;

THAT I believe I am the original, first, and sole inventor (if only one inventor is named below) or an original, first, and joint inventor (if plural inventors are named below or in an attached Declaration) of the subject matter which is claimed and for which a patent is sought on the invention entitled

IMMUNOGENIC COMPLEXES AND METHODS RELATING THERETO

(Attorney Docket No. 017227/0155)

the specification of which (check one)

___ is attached hereto.

XX was filed on February 17, 2000 as United States Application Number or PCT International Application Number _____ and was amended on February 17, 2000 (if applicable).

THAT I do not know and do not believe that the same invention was ever known or used by others in the United States of America, or was patented or described in any printed publication in any country, before I (we) invented it;

THAT I do not know and do not believe that the same invention was patented or described in any printed publication in any country, or in public use or on sale in the United States of America, for more than one year prior to the filing date of this United States application;

THAT I do not know and do not believe that the same invention was first patented or made the subject of an inventor's certificate that issued in any country foreign to the United States of America before the filing date of this United States application if the foreign application was filed by me (us), or by my (our) legal representatives or assigns, more than twelve months (six months for design patents) prior to the filing date of this United States application;

THAT I have reviewed and understand the contents of the above-identified specification, including the claim(s), as amended by any amendment specifically referred to above;

THAT I believe that the above-identified specification contains a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention, and sets forth the best mode contemplated by me of carrying out the invention; and

THAT I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I HEREBY CLAIM foreign priority benefits under Title 35, United States Code §119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application for patent or inventor's certificate or of

any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number	Country	Foreign Filing Date	Priority Claimed?	Certified Copy Attached?
PP8735/99	Australia	17 February 1999	Yes	
PQ1861/99	Australia	27 July 1999	Yes	

I HEREBY CLAIM the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below.

U.S. Provisional Application Number	Filing Date

I HEREBY CLAIM the benefit under Title 35, United States Code, §120 of any United States application(s), or § 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application Number	PCT Parent Application Number	Parent Filing Date	Parent Patent Number

I HEREBY APPOINT the following registered attorneys and agents of the law firm of FOLEY & LARDNER to have full power to prosecute this application and any continuations, divisions, reissues, and reexaminations thereof, to receive the patent, and to transact all business in the United States Patent and Trademark Office connected therewith:

STEPHEN A. BENT	Reg. No. 29,768
DAVID A. BLUMENTHAL	Reg. No. 26,257
BETH A. BURROUS	Reg. No. 35,087
ALAN I. CANTOR	Reg. No. 28,163
WILLIAM T. ELLIS	Reg. No. 26,874
JOHN J. FELDHAUS	Reg. No. 28,822
PATRICIA D. GRANADOS	Reg. No. 33,683
JOHN P. ISACSON	Reg. No. 33,715
MICHAEL D. KAMINSKI	Reg. No. 32,904
LYLE K. KIMMS	Reg. No. 34,079
KENNETH E. KROSIN	Reg. No. 25,735
JOHNNY A. KUMAR	Reg. No. 34,649
GLENN LAW	Reg. No. 34,371
PETER G. MACK	Reg. No. 26,001
BRIAN J. MC NAMARA	Reg. No. 32,789
SYBIL MELOY	Reg. No. 22,749
RICHARD C. PEET	Reg. No. 35,792

GEORGE E. QUILLIN	Reg. No.	32,792
COLIN G. SANDERCOCK	Reg. No.	31,298
BERNHARD D. SAXE	Reg. No.	28,665
CHARLES F. SCHILL	Reg. No.	27,590
RICHARD L. SCHWAAB	Reg. No.	25,479
ARTHUR SCHWARTZ	Reg. No.	22,115
HAROLD C. WEGNER	Reg. No.	25,258

and I request that all correspondence be directed to:

Stephen A. Bent
FOLEY & LARDNER
Washington Harbour
3000 K Street, N.W., Suite 500
Washington, D.C. 20007-5109

Telephone: (202) 672-5404
Facsimile: (202) 672-5399

I UNDERSTAND AND AGREE THAT the foregoing attorneys and agents appointed by me to prosecute this application do not personally represent me or my legal interests, but instead represent the interests of the legal owner(s) of the invention described in this application.

I FURTHER DECLARE THAT all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Name of first inventor	John Cooper COX
Residence	Victoria, Australia
Citizenship	Australian
Post Office Address	140 Dunns Road, Bullengarook, Victoria, Australia 3437
Inventor's signature	
Date	

Name of second inventor	Debbie Pauline DRANE
Residence	Victoria, Australia
Citizenship	Australian
Post Office Address	72 Mulcahy Road, Bullengarook, Victoria, Australia 3437
Inventor's signature	
Date	

SEQUENCE LISTING

<110> CSL LIMITED

<120> Immunogenic Complexes and Methods Relatng Thereto

<130> TDO

<140> International
<141> 2000-02-17

<150> PP8735/99
<151> 1999-02-17

<150> PQ1861/99
<151> 1999-02-17

<160> 9

<170> PatentIn Ver. 2.0

<210> 1
<211> 11
<212> PRT
<213> mammalian

<400> 1
Ser Leu Leu Met Trp Ile Thr Gln Cys Phe Leu
1 5 10

<210> 2
<211> 9
<212> PRT
<213> mammalian

<400> 2
Ser Leu Leu Met Trp Ile Thr Gln Cys
1 5

<210> 3
<211> 9
<212> PRT
<213> synthetic construct

<400> 3
Tyr Pro His Phe Met Pro Thr Asn Leu
1 5

<210> 4
<211> 36
<212> PRT
<213> synthetic construct

<400> 4
Tyr Pro His Phe Met Pro Thr Asn Leu Arg Pro Gln Ala Ser Gly Val
1 5 10 15

Tyr Met Thr Tyr Gln Arg Thr Arg Ala Leu Val Ser Tyr Ile Pro Ser
20 25 30

Ala Glu Lys Ile
35

<210> 5
<211> 9
<212> PRT
<213> synthetic construct

<400> 5
Arg Pro Gln Ala Ser Gly Val Tyr Met
1 5

<210> 6
<211> 9
<212> PRT
<213> synthetic construct

<400> 6
Thr Tyr Gln Arg Thr Arg Ala Leu Val
1 5

<210> 7
<211> 9
<212> PRT
<213> synthetic construct

<400> 7
Ser Tyr Ile Pro Ser Ala Glu Lys Ile
1 5

<210> 8
<211> 5
<212> PRT
<213> synthetic construct

<400> 8
Cys Asx Gln Cys Ala
1 5

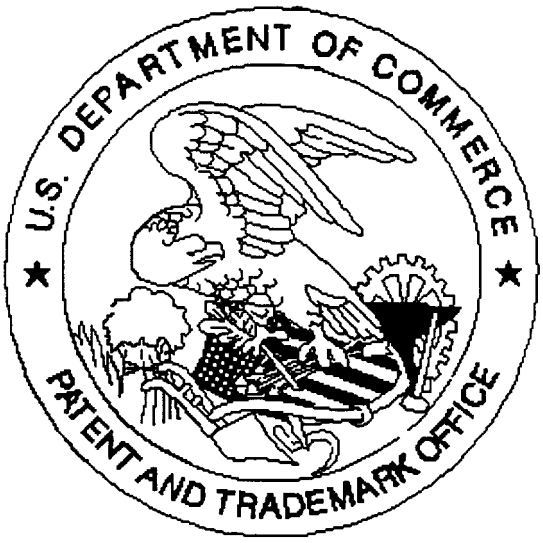
<210> 9
<211> 57
<212> PRT
<213> synthetic construct

<400> 9

```
Tyr Pro His Phe Met Pro Thr Asn Leu Thr Ser Ser Gly Pro Ser Asn
 1               5                  10                  15

Thr Pro Pro Glu Ile Phe Ala Pro Gly Asn Tyr Pro Ala Leu Ser Tyr
            20                  25                  30

Ile Pro Ser Ala Glu Lys Ile Glu Glu Gly Ala Ile Val Gly Glu Ile
        35                  40                  45

Arg Pro Gln Ala Ser Gly Val Tyr Met
    50                  55
```

United States Patent & Trademark Office

Office of Initial Patent Examination -- Scanning Division



SCANNED5

Application deficiencies were found during scanning:

☐ Page(s)_____ of _____ were not present for scanning. (Document title)

☐ Page(s)_____ of _____ were not present for scanning. (Document title)

There are 49 pages of specification, claims & abstract.

☐ Scanned copy is best available.